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**Evaluation of Berry Extracts on Intestinal Digestive Enzymes and Sugar
Transporters**

A Thesis Presented

by

JUNGBAE OH

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

Of the requirements for the degree of

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Nutrition

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Transporters**

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ABSTRACT

EVALUATION OF BERRY EXTRACTS ON INTESTINAL DIGESTIVE ENZYMES AND SUGAR TRANSPORTERS

MAY 2017

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Diabetes mellitus affects over 8% of the world population, with the great majority having type 2 diabetes mellitus (T2DM). T2DM is characterized by postprandial hyperglycemia and has been identified as key causes of morbidity and mortality with substantial health care costs due to its complications. Therefore, managing postprandial hyperglycemia can be an important and practical approach to reduce premature morbidity and mortality and economic loss. The causes of T2DM are multifactorial and include both genetic and environmental factors. Also, increased oxidative stress can be influenced on the prevalence of T2DM. However, sedentary lifestyle and high-calorie diets are considered as major contributing factors for the development of T2DM. In particular, consumption of high sucrose elevates blood glucose level dramatically. Thus, artificial sweeteners were extensively used, however artificial sweeteners are also increased the prevalence of T2DM. One of the therapeutic approaches to attenuate hyperglycemia is to inhibit intestinal α -glucosidase enzyme and suppress glucose transporters that regulate intestinal glucose digestion and absorption. In recent studies, consumption of fruits and vegetables has been shown to decrease postprandial

hyperglycemia and risk of T2DM by inhibiting carbohydrate digestion and absorption in the small intestine due to their bioactive compounds. Therefore, there is an increased interest to find the natural sources to prevent T2DM. In particular, demands of berry fruits are increased steadily due their natural antioxidants and bioactive compounds. The antidiabetic effects of various berry extracts or berry mixture in pre-clinical and clinical studies were shown. However, the underlying pathways of individual berries at the molecular level are still unclear. Also, it has not been investigated whether berries can manage the plasma glucose with dietary factors. It may be possible that beneficial effects of berry consumption could be through inhibiting carbohydrate digestion and absorption in the small intestine and it can be applied to dietary strategy so that postprandial hyperglycemia can be managed effectively.

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CHAPTER 1

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder and one of the major global health problems in the modern era [1]. According to CDC reports, 8.3% of the population already had diabetes in 2010 [2], and people with T2DM have about 2 to 4 times higher risk of other complications such as cardiovascular disease (CVD) or stroke in the United States [3]. A high caloric intake as a high-sugar and high-fat diet combined with a sedentary lifestyle has been identified as primary determinants in the development of obesity and T2DM [4]. In particular, a dramatic increase in the prevalence of obesity and T2DM has also been strongly correlated with sugar-sweetened beverages consumption in different population groups [5-8]. In recent years, non-calorie and non-digestible artificial sweetener (AS), sucralose, have been extensively used in a variety of food products as an alternative to simple sugars [9]. However, emerging evidence suggest that consumption of AS-contained beverages also increase the risk of T2DM and metabolic disorders [10, 11], which may be partially through upregulation of glucose absorption in the small intestine [12]. Although the global epidemic of obesity mostly explains the increased incidence and prevalence of T2DM, the precise mechanisms linking obesity to T2DM still remain unclear.

Among the characteristics, postprandial hyperglycemia is a hallmark of T2DM that has been recognized as an early defect in diabetic patients and prediabetic individuals [13]. Postprandial hyperglycemia can be caused by decreased glucose utilization in peripheral tissues, resulting in insulin resistance [14]. It can also result from increased rate of intestinal digestion and absorption from dietary carbohydrates [15]. Therefore, identifying dietary bioactive components that can delay carbohydrate digestion and

absorption in the small intestine to prevent postprandial hyperglycemia is crucial for the management of T2DM [16].

One of the primary targets of antidiabetic drugs such as acarbose is to inhibit intestinal carbohydrate digesting enzymes and sugar transport proteins, which have been demonstrated to be effective in reducing hyperglycemia after a meal [17]. However, these anti-diabetic drugs are often reported to cause several side effects such as nausea, vomiting, abdominal cramps [18] or urinary tract infections [19]. Therefore, there has been increasing interest in investigating dietary bioactive constituents from natural sources that may modulate carbohydrate digestion and absorption in the small intestine.

Among natural sources, fruits and vegetables have many beneficial nutrients and phytochemicals that are thought to protect against T2DM [20, 21]. In particular, demands of cranberries among consumers have been increased due to valuable bioactive compounds [22] that reduce the risk of CVD [23, 24] and urinary tract infection [25]. Recent research has also shown an anti-diabetic potential of cranberries in both *clinical* studies [26] and *in vivo* animal studies [27]. Antidiabetic properties of cranberry consumption may result from delayed digestion and absorption of carbohydrates in the small intestine. Several *in vitro* studies reported that cranberry extracts and its bioactive compounds inhibit yeast α -glucosidase activity [28] as well as rat α -glucosidase activity [29]. In addition, berry mixtures prepared from several berries including cranberries have also shown decreased glucose uptake by inhibiting glucose transporters in Caco-2 intestinal cells [30].

Among many different types of berries, blackberry extracts have been reported to have hypoglycemic effects in animal studies [31, 32] and *in vitro* studies, where blackberries inhibited yeast α -glucosidase activity [33]. However, postprandial anti-hypoglycemic

effects of blackberries have not been studied in *in vivo* settings.

Dietary polyphenols have received immense attention among nutritionists, researchers, and consumers due mainly to their important roles in human health such as disease prevention and health promotion. Among the wide range of polyphenols, the most abundant polyphenols present in berries are anthocyanidins, proanthocyanidins, flavanols, and phenolic acids [34]. Those biological substances in berries have been demonstrated to have health benefits in humans with strong antioxidant and anti-inflammatory activities [35-37]. Furthermore, recent research has shown an antidiabetic potential of dietary berry polyphenols by inhibiting glucose uptake in human Caco-2 intestinal cells [30, 38]. This may indicate that quantity as well as compositions of berry polyphenols is important to achieve health benefits. While there is some evidence from the published studies that cranberries and blackberries or their bioactive compounds inhibit both yeast and rat α -glucosidase enzyme activity, it is currently unclear whether cranberries and blackberries exert inhibitory effects on α -glucosidase activity in human cell models of intestinal absorptive function such as Caco-2 intestinal cells. Moreover, the underlying molecular mechanisms of individual berries on intestinal carbohydrate digestion and absorption are not well understood. Although several studies have evaluated the antidiabetic potential of various berries, dietary conditions such as in the presence of dietary carbohydrates and AS were not considered. Therefore, it is not known whether cranberries and blackberries may suppress intestinal digestion and absorption of carbohydrates in the presence of various dietary carbohydrate sources.

The overall goal of this study was to identify potential role of berries as part of a dietary strategy and molecular mechanisms so that postprandial hyperglycemia can be managed effectively and the need for pharmacotherapy could be greatly reduced. The aims of the study were (1) to determine the extent to which cranberry extracts (CBE)

and blackberry extracts (BBE) regulate intestinal α -glucosidase and/or gene expression of glucose transporters; (2) whether this correlates with the level of glucose uptake using human Caco-2 intestinal cells; and (3) investigate whether CBE and BBE modulate intestinal α -glucosidase and glucose transporters in the presence of dietary carbohydrates and AS.

CHAPTER 2

LITERATURE REVIEW

2.1 Trends of type 2 diabetes (T2DM): Global epidemic

T2DM is a metabolic disorder of fat and glucose metabolism and is the most common form of diabetes [1]. The estimated worldwide prevalence of T2DM has continued to increase dramatically during the past two decades [39]. According to World Health Organization (WHO), the prevalence of diabetes in adult population worldwide is 8.5% in 2014 and is about two times higher compared to 1980 [40]. In addition, it is estimated that over 360 million people would have T2DM by the year 2030 [41].

The rising prevalence of T2DM is especially notable in low and middle-income countries [42]. For example, the prevalence of T2DM in developing countries in Asia has increased three to five folds during the past 30 years and now accounts for about 60% of the global diabetic population. As this trends will continue to increase at this rate, this will certainly be a major global health issue [43]. While the rise of T2DM has been mostly observed in the adult population, increasing prevalence of T2DM in youth will become a serious new T2DM epidemic in the 21st century [44]. Until 1990, the prevalence of T2DM was rarely seen in childhood. However, the T2DM prevalence rate in youth has been significantly increased within ten years in many other countries [45]. Such dramatic increases of T2DM in low and middle-income class and young ages are strongly linked to changes in lifestyles with high-calorie diets such as high carbohydrates and high fat [4].

2.1.1 Diabetic complications & economic burden

Diabetes and its complications affect many different organ systems in the body over a period of time and are a major cause of morbidity and mortality with substantial health care costs [3]. According to National Health and Nutrition Examination Survey (NHANES), chronic kidney disease showed the highest prevalence rates (27.8 %), followed by foot problems (22. 9%) and eye damages (18. 9%), which are classified as a microvascular disease. While the prevalence rate of microvascular accounts for around 70%, the mortality rate of diabetic complications is mainly due to macrovascular disease such as heart disease and stroke, which accounts for 9.8% and 6.6%, respectively [46].

The burden of diabetes is not only on the health, but also on total health care cost. According to the American Diabetes Association (ADA), direct and indirect health care costs for diabetes is estimated over 240 billion dollars in 2012 [47]. Notably, approximately 40% of the total cost of diabetes is due to treatment of diabetes complications, and it is estimated that diabetes-related macrovascular disease accounted for 85% of cumulative costs of diabetic complications [48].

Among the risk factors, hyperglycemia has been identified as a key diagnostic measure of T2DM and also as major diabetes-mediated health complications. [49, 50]. Thus, improvement of postprandial hyperglycemia has been effective in attenuating the macrovascular complications in diabetic patients [51-53]. As a consequence, managing postprandial hyperglycemia can be an important and practical approach to reduce premature morbidity and mortality as well as economic burdens.

2.2 Pathophysiology of T2DM

The causes of T2DM are multifactorial and include both genetic and environmental factors that β -cell function and insulin sensitivity in peripheral tissue [54]. Even though the numbers of genomes are associated with the prevalence of T2DM, a relatively small percentage is due to monogenic causes [55]. Epidemiological and experimental evidence supported that environmental factors including obesity, sedentary lifestyle, and high-calorie diets are major contributing factors for the development of T2DM [56-58]. Notably, high carbohydrates and high fat diets have been shown to induce T2DM by a combination of pancreatic β -cell dysfunction and insulin resistance in peripheral tissues [56].

Under normal physiological conditions, postprandial plasma glucose concentrations are regulated by the actions of hormones, mainly insulin and glucagon [59]. In the fed state, pancreatic islet β -cells secrete insulin in response to plasma glucose level. As a consequence of insulin secretion, plasma glucose is transported into insulin-sensitive peripheral tissues via glucose transporters and anabolic metabolism is promoted. Furthermore, glucagon secretion is inhibited so that lipolysis in adipose tissue and hepatic gluconeogenesis are suppressed [56].

On the other hand, diets high in carbohydrates and fat cause substantial hyperglycemia, resulting in increased peripheral tissues insulin resistance and islet β -cells dysfunction [60, 61]. In addition, the insulin resistance state is an impairment in the insulin-mediated suppression of lipolysis and increased secretion of pro-inflammatory cytokines in adipose tissue, which in turn aggravate insulin resistance in muscle and liver. [62]. Numerous studies have demonstrated that elevated plasma free fatty acids (FFAs) as a result of increased adipose lipolysis impair insulin-stimulated glucose

uptake in skeletal muscle. Similarly, elevated FFAs also stimulate hepatic gluconeogenesis, and endogenous glucose production, which results in decreased hepatic insulin action with increase the severity of insulin resistance [63].

While reduced glucose utilization by peripheral tissues in insulin resistant state elevates plasma glucose level, postprandial hyperglycemia can also be directed affected by types and composition of dietary carbohydrates as well as their intestinal digestion [16]. One important therapeutic approaches for treating postprandial high blood glucose in T2DM is to reduce digestion and absorption of dietary carbohydrates. These approaches can be accomplished by inhibiting carbohydrate hydrolyzing enzymes in the small intestine. In humans, α -1-4 glycosidic linkage of complex carbohydrates is digested into disaccharides and oligosaccharides by the action of salivary and pancreatic α -amylase. Then, disaccharides are further hydrolyzed into monosaccharides by the action of two subunits of α -glucosidase, sucrase-isomaltase (S-I) and maltase-glucoamylase (M-G), in the apical membrane of the small intestine [64].

Absorption of monosaccharides involved transport from the intestinal lumen into epithelial cells. The glucose and galactose are carried by the sodium-dependent glucose transporters 1 (SGLT1), while fructose is carried by the glucose transporter 5 (GLUT5). Glucose, galactose, and fructose are then transported out of the enterocyte via another hexose transporter, glucose transporter 2 (GLUT2) across basolateral membrane and into blood [65]. Recent in vivo animal studies suggested that GLUT2 can also be recruited into the apical side of membrane when the luminal glucose concentration is high such as occurs in the fed state, enhancing facilitated diffusion [65]. Thus, retardation of intestinal glucose absorption by inhibiting intestinal glucose transporters has been targeted to reduce postprandial hyperglycemia.

2.3 Role of oxidative stress & antioxidant in metabolic disorders & diabetes

Free radicals are reactive atoms that have one or more unpaired electrons that are associated with many cellular process and human disease, depending on concentration. [66]. In the human body, oxidants or reactive oxygen species (ROS) including hydroxyl ($\text{OH}\bullet$), superoxide ($\text{O}_2\bullet^-$) are generated as a consequence of a variety of normal physiological metabolism and particularly, cellular oxidative phosphorylation in the mitochondria [67, 68]. At low concentrations, oxidants or ROS during normal physiological processes, it acts as stimuli for cell signaling, however, excessive of ROS or oxidants can react with lipids, proteins, and nucleic acids, causing cellular damages or apoptosis, and even diabetes and other metabolic complications [67].

Free radicals produced from normal physiological metabolisms are effectively neutralized by non-enzymatic and enzymatic antioxidants to prevent the propagation of free radical reactions or oxidants, maintaining the balance of redox system [69]. The endogenous antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD is located in the cytosol (Cu,Zn-SOD) and mitochondria (Mn-SOD) and converts superoxide anion radicals into oxygen and less toxic hydrogen peroxide [70]. The enzyme Cat present in the peroxisomes further decomposes hydrogen peroxide into oxygen and water. In addition, the enzyme GPx degrades both hydrogen peroxide and lipid hydroxy peroxides in coordination with glutathione reductase in the presence of NADPH as a cofactor [71].

Recent studies suggest that postprandial hyperglycemia causes overproduction of superoxide anions via mitochondrial electron transport chain which exceeds endogenous antioxidant capacity and shift into oxidative stress [49]. Endogenous

defense system becomes weakened and unable to counteract the increased ROS, causing tissue damage through multiple mechanisms. Cellular injury by ROS from hyperglycemia involves enhanced polyol pathway and increased cellular formation of advanced glycation end products (AGEs), which are known to activate protein kinase (PKC) and NF- κ B, key mediators of islet β -cell dysfunction [72]. Therefore, consumption of exogenous antioxidants has been emphasized to maintain the oxidant-antioxidant balance in our body.

2.4 Oral anti-hyperglycemic therapies for Type 2 diabetes

Diet modification with regular physical activities is recommended for preventing the prevalence of T2DM and is central concepts of any therapeutic programs. Emerging studies suggested that a significant reduction in the incidence of T2DM through lifestyle and diet modifications [58, 73]. Because of elevated fasting and postprandial blood glucose that expose patients to acute and chronic health complications, pharmacological therapies are mainly targeting glycemic control by the drugs that increase glucose utilization in peripheral tissues or suppress digestion and absorption of dietary carbohydrates in the small intestine [74]. Some of the available pharmacological agents include Sulfonylureas, Biguanides, Thiazolidinedione, intestinal α -glucosidase inhibitors, and intestinal glucose transporter inhibitors as discussed below.

2.4.1 Sulfonylureas

Sulfonylureas are the oldest antidiabetic agents and are commonly prescribed for the treatment of T2DM patients [75]. The most significant effect of sulfonylureas is to suppress glucagon secretion from pancreatic α -cells and increase plasma levels of

insulin secreted from pancreatic β -cells, even though extra pancreatic actions may also contribute to their anti-hyperglycemia effects [76]. Thereby, treatment with sulfonylureas decreases plasma glucose and HbA1c level [74]. However, stimulation of endogenous insulin secretion via sulfonylureas often causes loss of efficacy over time, which appears to be related to exhaustion of β -cell function. Hypoglycemia and weight gain are the two frequently observed side effects of these drugs. In particular, elderly T2DM patients have over 30% increased the risk of hypoglycemia than younger patients so that sulfonylureas therapy should be carefully monitored avoided in elder people with diabetes [77].

2.4.2 Biguanides

Metformin is one of the biguanides that commonly prescribed for T2DM treatment for long period of time [78]. The precise mode of action of metformin is not fully understood, but predominant effects of metformin are to suppresses hepatic glucose production through inhibition of gluconeogenesis [79]. Also, metformin acts as an insulin-sensitizer that stimulates glucose uptake in peripheral tissues such as skeletal muscle [80] and decreases the absorption of glucose from the gastrointestinal track. Therefore, metformin reduces postprandial hyperglycemia without promoting weight gain. [81]. Side effects associated with metformin therapy are gastrointestinal complaints and lactic acidosis [82] so that care should be taken from those affected individuals.

2.4.3 Thiazolidinedione (TZD)

TZDs is one of the oral antidiabetic drugs that improves glycemic control in T2DM patients through the improvement of insulin sensitivity in peripheral tissues [83]. TZDs are synthetic ligands for a nuclear receptor, peroxisome-proliferator-activated receptor

gamma (PPAR γ), and the most prominent effect of TZDs is thus mediated by PPAR γ through stimulation of glucose into skeletal muscle and adipose tissue, a process which requires insulin [84]. The major side effects of TZDs are primarily associated with weight gain and edema. In some cases, TZDs are reported to increase the risk of heart failure in patients with T2DM [85]. Because of these reported adverse events, the therapy needs to be prescribed with caution and more studies are in needed for its safety.

2.4.4 Intestinal α -glucosidase inhibitors (AGIs)

Acarbose, Voglibose, and Miglitol are α -glucosidase inhibitors (AGI) that have been prescribed for managing postprandial glucose level in pre-diabetic and diabetic patients [86]. AGIs act as competitive and reversible inhibitors of the α -glucosidase enzyme, which delay intestinal digestion and absorption of carbohydrates and mitigate rapid spike of plasma glucose level after a meal [87]. AGIs are considered to be safe and effective because it is rarely absorbed in our body. Due to AGIs regulation of postprandial hyperglycemia, AGIs have been shown a significant reduction in the risk of CVD and hypertension in clinical studies [88]. Moreover, unlike sulfonylureas, AGIs do not affect plasma insulin levels. As a result, they do not cause hypoglycemia when used alone. These agents are not usually used for primary therapy and most useful in combination with other drugs. Gastrointestinal side effects are common and the major complaints are bloating, flatulence, diarrhea, abdominal discomfort, and pain [18].

2.4.5 Intestinal glucose transporter inhibitors

SGLT1 is essential for intestinal glucose and galactose absorption and inhibitors such as sotagliflozin (LX4211) have been reported to hold promise in improving glycemic control by retarding postprandial glucose peaks in T2DM [19]. The efficacy of sotagliflozin has been encouraging as it effectively reduces blood HbA1c and glucose

levels, suggesting potential efficacy in T2DM [89]. In particular, a dual inhibitory action of sotagliflozin on intestinal SGLT1 and SGLT2 would provide unique insulin-independent mechanisms to treat T2DM [89]. Hypoglycemia and urinary track infections are notable side effects due to increased delivery of sugars to the distal region of the small intestine and colon [19]. While promising from a clinical profile of postprandial plasma glucose reduction, longer term studies with larger sample sizes are needed to fully characterize the efficacy and safety of the drugs.

2.5 Role of diet & dietary components in diabetes

Recent epidemiological studies suggested that dramatic increases in obesity and T2DM have been observed in different population groups where consumed high fat and carbohydrate diets including sugar-sweetened beverage [5-8]. Thus, reducing carbohydrate and fat-rich meal have been shown to reduce postprandial glucose response in individuals with T2DM [57, 90, 91].

Artificial sweeteners (ASs) are considered safe and beneficial owing to their non-caloric and non-digestible characteristics. Therefore, AS has been extensively used in variety food products as an alternative to sugars [9]. Although the impact of ASs on metabolic diseases is controversial, recent evidence clearly suggested that consumption of ASs especially artificially sweetened beverages increased the risk of T2DM and metabolic disorders [10, 11, 92, 93]. The molecular mechanisms of AS on metabolic disorder and T2DM are yet to be investigated. However, it has been reported that ASs increase postprandial hyperglycemia by increasing intestinal glucose transporters [12].

For individuals with T2DM, a recommended diet high in fruits, vegetables, and dietary fiber has proven beneficial in lowering markers related to oxidative stress as well as in

managing postprandial hyperglycemia. Fruits and vegetables are primary dietary sources many essential nutrients and phytochemicals that are recognized to prevent or delay the risk of T2DM [20, 21]. A variety of phenolic compounds including the family of flavonoids, flavanols, flavanones, flavones, and anthocyanidins are also found in the fruits and vegetables [94], which are also abundant source of vitamins and minerals [95]. The antioxidant properties of fruits and vegetables are due to a chemical structure of phenolic compounds and aromatic phenolic rings that are able to donate hydrogens to stabilize free radicals [96]. In addition, phytochemicals in fruits and vegetables have been shown to inhibit intestinal α -glucosidase activity[97], delaying carbohydrate digestion and absorption into body [98]. Therefore, primary dietary intervention strategies employ increased consumption of fruits and vegetable rich in dietary fibers.

2.6 Berries and their roles in health & diabetes

Berry fruits are a rich source of natural antioxidants and also high contents of bioactive compounds including ascorbic acid, anthocyanins, phenolic acids, flavanols, flavonols and tannins [99-101]. Due to high amounts of bioactive and health-promoting compounds, berries are widely recognized as functional products that provide health benefits beyond basic nutrition as well as prevent metabolic disorders.

Several intervention studies have investigated the effects of different berries on CVD in healthy humans or people with CVD risk factor. Those studies suggested that berry fruits significantly decrease postprandial oxidative stress and low-density lipoprotein oxidation, suggesting that berry intake may prevent the incidence of CVD. [102-105]. Currently, the impacts on glucose metabolism have been limited although recent *in vitro* studies indicate potential effects of berry extracts at the level of the small intestine.

Recent evidence demonstrated that consumption of berries or berry mixture significantly reduced postprandial hyperglycemia in healthy individuals [106-109]. For example, bilberry extracts have been reported to ameliorate hyperglycemia and insulin sensitivity in T2Dm mice via activation of AMP-activated kinase (AMPK) [110]. In addition, various berry extracts and polyphenols derived from berries can inhibit starch digestive enzymes and glucose transporters in the gastrointestinal track *in vitro* [111]. Many studies so far have investigated the effects of bioactive polyphenols isolated from berries or berry mixture on starch digestive enzymes and glucose transporters. It is thus possible that beneficial effects of berry consumption could be through there and/or other cellular targets that may aid in reducing postprandial glucose levels and enhancing insulin sensitivity, which could support the use of berries or berry extracts to manage metabolic disorders. Further studies of the effect of berry consumption on gene expression are valuable to identify potential mechanisms of action.

2.6.1 Cranberries

The America cranberries (*Vaccinium macrocarpon*) is one of the berries commonly consumed in America, and over 90% of global cranberries are produced in global [112]. Cranberries have been known to be rich sources of phytochemicals. Currently, over 150 individual phytochemicals including anthocyanins have been identified [113]. Several human studies provide evidence for effect of cranberry bioactive on chronic disease. In particular, cranberries are well known to reduce the risk of urinary tract infections [114, 115] and CVD [23, 24] that led to the inclusion of cranberries to modifications. Also, clinical and animal studies from recent years have revealed new health benefits of cranberry consumption on T2DM prevention although effects of cranberries on postprandial hyperglycemia are currently limited. It has been shown that consumption of raw cranberries exhibits a favorable response to plasma insulin and glucose levels in

people with T2DM [26], and high fructose diet with cranberry powder for 11 weeks tend to reduce postprandial hyperglycemia in an animal model [27]. Antidiabetic properties of cranberries may result from delaying intestinal digestion and absorption of carbohydrates. There is an evidence showing that cranberries inhibit yeast α -glucosidase activity [28] and a bioactive compound in cranberries, Cyanidin-3-galactoside, also suppresses rat α -glucosidase activity [29]. In addition, in both short and long-term exposure of berry mixture, which derived from several berries including cranberries, decreased two main glucose transporters, SGLT1 and GLUT2, as well as glucose uptake in human Caco-2 intestinal cells [30]. While a body of evidence suggest that glucose absorption across the apical membrane of enterocyte is hindered by berry polyphenols, the precise contribution of specific bioactive compounds and individual berries has not been studied. These further studies will warrant identification of important modulators of the rate of glucose digestion and absorption and thus of plasma blood glucose levels.

2.6.2 Blackberries

Blackberry (*Rubus sp.*) is known to have high contents of phenolics including anthocyanins and ellagitannins, which contribute to potent antioxidant properties and other biological benefits of blackberries [116]. *In vitro* studies have shown that blackberry extracts exert inhibitory effects of A549 human lung cancer with reduced neoplastic transformation in normal epidermal mouse cells [117]. Furthermore, blackberry extracts have the dose-dependent anti-proliferate effects in various cancer cell lines including human oral, breast, prostate, and colon cancer cell line [117]. While anticarcinogenic properties of blackberries have been demonstrated in several *in vitro* studies, possible health benefits against such metabolic disorder like blood glucose regulation and T2DM have not been extensively studied. In particular, it has not been

studied in relation to postprandial hyperglycemia in a clinical study. However, limited evidence showed that dietary supplementation of blackberry extracts in rats for 80 days decreased plasma glucose concentration and increased peripheral tissue insulin sensitivity [31]. Also, blackberry intake significantly decreased fasting blood glucose level on diabetic rats [32]. These results indicate that hypoglycemic effects of blackberries may contribute to decreased the risk of T2DM and it may result from inhibition of intestinal α -glucosidase activity, which was shown *in vitro* study [33].

CHAPTER 3

BERRY EXTRACT EXERT ANTI-DIABETIC EFFECTS VIA INHIBITION OF α -GLUCOSIDASE AND GLUCOSE TRANSPORTERS IN HUMAN INTESTINAL EPITHELIAL CACO-2 CELLS

3.1 Abstract

T2DM is a chronic disease characterized by postprandial hyperglycemia. One of the therapeutic approaches to attenuate hyperglycemia is to inhibit intestinal α -glucosidase enzyme and/or suppress glucose transporters that regulate intestinal glucose transporters such as SGLT1 & GLUT2. Berries rich in polyphenol antioxidants have various health benefits. Although the antidiabetic effects of various berry extracts or berry mixture in *pre-clinical* and *clinical* studies, the underlying pathways at the molecular level is still unclear. In this study, we investigated antioxidant and antidiabetic effects of selected berry extracts by determining free radical scavenging activities, Caco-2 intestinal α -glucosidase activity, glucose uptake and the gene expression of α -glucosidase and glucose transporters in Caco-2 cells. Total phenolic contents of berry extracts varied from 28.55 ± 0.06 to 56.15 ± 1.08 gallic acid equivalent (GAE $\mu\text{g/mL}$) and correlated with antioxidant capacities. Both cranberry extract (CBE) and blackberry extract (BBE) at 200 $\mu\text{g/mL}$ concentration significantly decreased glucose uptake in Caco-2 cells. While mRNA expression and activity of α -glucosidase were inhibited by CBE and BBE, mRNA expression of SGLT1 and GLUT2 was only inhibited by CBE. Moreover, CBE and BBE significantly decreased glucose uptake in the presence of sucrose and AS. Our data suggest that CBE and BBE have different molecular mechanisms in suppressing hyperglycemia and their effects are mediated by

inhibiting carbohydrate digestion and absorption.

3.2 Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia and has been steadily increased during the past two decades worldwide [38]. Furthermore, people with T2DM have about 2 to 4 times higher risk of health complications such as cardiovascular disease (CVD), which is one of the major health problems in the 21st century [3]. Among the features of T2DM, postprandial hyperglycemia is a hallmark of clinical manifestations and a major contributor to metabolic complications [118]. Therefore, controlling postprandial hyperglycemia is recognized as a prime clinical strategy for the prevention of T2DM and related cardiovascular events [119].

As postprandial hyperglycemia is primarily regulated by the rate of intestinal digestion and absorption of carbohydrates [16], one of the therapeutic approaches for postprandial hyperglycemia is to retard intestinal digestion and absorption processes. In humans, complex dietary carbohydrates are digested into monosaccharides by the action of α -amylase and α -glucosidases, mainly sucrase-isomaltase (S-I) and maltase-glucoamylase (M-G), in the apical membrane of the small intestine [64]. Individual monosaccharides are then transported into enterocytes through glucose transporters, mainly sodium-dependent glucose transporter 1 (SGLT1) and non-sodium dependent glucose transporter 2 & 5 (GLUT2, GLUT5). Then, basolateral GLUT2 transports monosaccharides from enterocytes to the portal circulation [65]. Several epidemiological studies have shown a strong association between high carbohydrate diets and sugar-sweetened beverages with hyperglycemia and T2DM [5-8]. Furthermore, non-digestive

and non-absorbable artificial sweeteners (AS), have been extensively used as alternatives to simple sugars that are shown to increase the risk of T2DM in clinical studies [10, 11]. A possible mechanism may involve upregulation of glucose absorption in the small intestine [12]. Therefore, diet modification with reduced consumption of dietary sugars or AS has been emphasized to help manage postprandial hyperglycemia.

One of the pharmacological approaches to hyperglycemia and T2Dm is to modulate the intestinal digestion and absorption of dietary complex carbohydrates, decreasing postprandial blood glucose levels. There are several currently available drugs including agarose, an inhibitor of intestinal α -glucosidase enzyme and sotagliflozin, an inhibitor of intestinal SGLT1. These drugs are designed to reduce glucose absorption in the GI track, and have been shown to be effective in reducing hyperglycemia after a meal [86, 89]. However, these antidiabetic drugs often cause some side effects including flatulence, abnormal discomfort, diarrhea [18], and urinary tract infections [19]. Therefore, there have been increasing interests in finding dietary bioactive constituents from natural sources that can delay carbohydrate digestion and absorption in the small intestine without such adverse effects.

It is well recognized that hyperglycemia induces overproduction of reactive oxygen species (ROS), which is known to cause both microvascular and macrovascular complication of diabetes [49]. Elevated oxidative stress also increases the formation of advanced glycation end-products (AGEs), causing islet β -cell dysfunction via PKC and NF- κ b pathways. [72]. Therefore, maintaining the balance between antioxidant and oxidant stress by dietary antioxidants is crucial to prevent hyperglycemia-mediated health complications.

Phytochemicals that are thought to protect against postprandial hyperglycemia in

T2DM [20, 21] and one proposed mechanism is through retardation of glucose uptake at the small intestinal level [30, 38]. The consumption of berry fruits and their health benefits is a matter of considerable interest. In particular, cranberries have received much attention from the public due to many health benefits. Recent research has shown antidiabetic potential of cranberries in both human [26] and animals [27]. Several *in vitro* studies also reported that cranberry extracts or its bioactive compounds inhibit yeast α -glucosidase activity [28] as well as rat α -glucosidase activity [29]. In addition, berry mixture containing cranberries showed potency of retardation of glucose absorption of in Caco-2 intestinal cells [30]. Therefore, antidiabetic properties of cranberries may result from delayed digestion and absorption of carbohydrates in the small intestine. Among different types of berries, blackberries have been reported to have higher antioxidant capacity than cranberries [120] and exert hypoglycemic effects in animal studies [31, 32]. Using *in vitro* models, it was shown that blackberry cultivars inhibit α -glucosidase activity, which may account for hypoglycemic effects *in vivo* [33]. However, clinical evidence of postprandial hypoglycemic effects of blackberries have not been studied.

While cranberries and blackberries have shown the inhibitory effects on both yeast and rat α -glucosidase enzyme activity, it is currently unclear whether cranberries and blackberries exert the inhibitory effects on α -glucosidase activity in human Caco-2 intestinal cells and whether the effects are related to total phenolic contents. Moreover, the underlying molecular mechanisms of individual berries on intestinal carbohydrate digestion and absorption are not well understood. The published studies evaluated potential efficacies of various berries without considering dietary factors such as sucrose and AS. Therefore, we evaluated antidiabetic effects by determining antioxidant capacity and total phenolic contents of berry extracts as well as rat and

Caco-2 α -glucosidase activities. In addition, effects of berry extracts on gene expression of carbohydrate digesting enzymes and glucose transporters are evaluated in the presence of sucrose or AS as dietary strategies in the prevention and treatment of postprandial hyperglycemia and T2DM.

3.3 Materials and methods

Freeze-dried blackberry, strawberry, raspberry, blueberry, and cranberry powders were purchased from (North Bay Trading Co. (Brule, WI, USA). 2,2-Diphenyl-1-picrylhydrazyl was purchased from Alfa Aesar. (Ward Hill, MA, USA). Folin-Ciocalteu phenol reagent, rat intestinal acetone powders, 4-nitrophenyl α -d-glucopyranoside reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium acetate, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), Iron (III) chloride hexahydrate were purchased from Santa Cruz Biotechnology. (Dallas, TX, USA). 6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (6-NBDG) was purchased from Cayman Chemical (Ann Arbor, MI). The fast SYBR real-time PCR master mix, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics, and trypsin-EDTA were purchased from Life Technologies (Grand Island, NY, USA). The specific primers for quantitative real-time PCR were designed by Primer Quest program, and purchased from integrated DNA Technologies (Coralville, Iowa, USA). Unless noted otherwise, all chemicals were purchased from Fisher Scientific Company Co. (Bridgewater, NJ, USA).

3.3.1 Sample preparations

The extraction procedure was carried out according to the method of Michiels et al.

[121] with slight modifications. Freeze dried berry powders were extracted with solvent mixture (Acetone/water/acetic acid; 70:29.5:0.5 v/v) for 1 hr at room temperature in dark place by using multi vortexer at 1200 rpm. Berry extracts were then filtered with Whatman filter paper No.3. Remained acetone from sample extracts was removed and concentrated under reduced pressure at 35°C by using the rotary evaporator. The freeze-dried powders were stored at – 80 °C

3.3.2 Cell culture

Human intestinal Caco-2 cells (passage number 75-79) were seeded into cell culture plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37°C in an atmosphere of 5% of CO₂, and media was replaced every 48 hrs. The Caco-2 cells were treated with samples at day 8 after confluence. In order to evaluate the effect of the berry extracts in presence of dietary sucrose or AS, cells were preincubated in DMEM without 10% of fetal bovine serum for 3 hrs prior to sample treatments.

3.3.3 Total phenolic contents

Total phenolic contents were determined following the procedure previously described by Blainsket al. [122] with a slight modification. 1 mL of sample was mixed with 10% (v/v) Folin-Ciocalteu phenol reagent and 2% Na₂CO₃ at the ratio of 1:1:1. The mixture was placed in dark place for 60 min and the observance was measured at 750 nm using Micro-Plate Reader (Molecular devices; Sunnyvale, CA, USA). The amounts of phenolic compounds were expressed as gallic acid equivalent (GAE µg/mL). Standard curve was obtained using various concentrations of gallic acid dissolved in water.

3.3.4 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activities

The antioxidant capacity was determined by DPPH radical scavenging method as described by Tailor et al. [123] with some modifications. A sample solution (40 μ L) was mixed with 160 μ L of 400 mM DPPH solution in 95% ethanol. Then, the mixture was incubated for 10 min at room temperature. Absorbance was measured at 517 nm by a Micro-Plate Reader and compared with controls which contained 40 μ L of 50% DMSO used to dissolve the extract powders. The DPPH radical scavenging activity was expressed as percent inhibition and calculated based on the formula as follows:

$$\text{Inhibition (\%)} = \left[\frac{\Delta A_{\text{Control}}(517\text{nm}) - \Delta A_{\text{Extract}}(517\text{nm})}{\Delta A_{\text{Control}}(517\text{nm})} \right] \times 100$$

3.3.5 Ferric reducing ability of plasma (FRAP) assay

Ferric reducing ability of plasma was determined following the procedure previously described by Benzine et al. [124] with slight modification. FRAP reagent was made with 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) and 20 mM Iron (III) chloride hexahydrate with 10:1:1 ratio. TPTZ and Iron (III) chloride hexahydrate were dissolved in 40 mM hydrogen chloride before the experiment. 1.9 mL of FRAP reagent was mixed with in 100 μ L of sample and incubated at room temperature for 30 min. The absorbance was measured at 593 nm by a Micro-Plate Reader and compared with controls which contained 50% DMSO. Ferric reducing ability of plasma was expressed as ascorbic acid equivalent (AAE μ M/mL). Standard curve was obtained using various concentrations of ascorbic acid.

3.3.6 Nitric oxide (NO) assay

Nitric oxide was determined following the procedure previously described by Tsikas et al. [125] with slight modification. 1 mL of sodium nitrite (10 mM) was mixed with 1 ml sample and pH was adjusted to 1.2 in the final volume of 10 mL. The mixture was then incubated at 37 °C for 1 h. After one hour, 0.4 ml of Griess reagent and 1 mL of mixture were mixed with 2.5 mL of 2% acetic acid. The mixture was then incubated for 15 min until pigment was expressed. The absorbance was measured at 520 nm by a Micro-Plate Reader and compared with controls which contained 1 mL of 50% DMSO. The nitric oxide inhibitory activity was expressed as percent inhibition and calculated based on the formula as follows:

$$\text{Inhibition (\%)} = \left[\frac{\Delta A_{\text{Control}}(520\text{nm}) - \Delta A_{\text{Extract}}(520\text{nm})}{\Delta A_{\text{Control}}(520\text{nm})} \right] \times 100$$

3.3.7 Rat α -glucosidase activity

Rat intestinal α -glucosidase activity was determined using the substrate, 5 mM of p-nitrophenyl- α -D-glucopyranoside (pNPG), as demonstrated by Kwon et al. [36] with a slight modification. Rat-intestinal acetone powder suspending in 0.9% of saline and vortexed for 6 min at 4 °C, centrifuging (13,000 \times g, 15 min, 4 °C), and supernatant was collected for the experiment. 100 μ L of rat intestinal α -glucosidase solution (1.0 U/mL) was then mixed with 50 μ L of sample solution and 50 μ L of substrates or buffer, and was the mixture further incubated at 37 °C for 30 min. After incubation, Na₂CO₃ was added to stop the reaction. The absorbance was recorded at 405 nm by Micro-Plate Reader and compared with controls. The rat α -glucosidase inhibitory activity was

expressed as percent inhibition and calculated based on formulation as follows:

$$\text{Inhibition (\%)} = \left[\frac{\Delta A_{\text{Control}}(405\text{nm}) - \Delta A_{\text{Extract}}(405\text{nm})}{\Delta A_{\text{Control}}(405\text{nm})} \right] \times 100$$

3.3.8 Caco-2 α -glucosidase activity

Caco-2 α -glucosidase activity was determined following the procedure previously described by Yu et al. [126]. Caco-2 cells were seeded into 6-well plates and collected with phosphate buffered saline (PBS) at 8 day after confluence. Caco-2 lysate (100 μ L), sample solution (50 μ L), and 5 mM pNPG solution or PBS were placed in 96-well plates. The mixture was then further incubated at 37 °C. After incubation, Na_2CO_3 was added to stop the reaction. The absorbance was measured at 405 nm using Micro-Plate Reader. The Caco-2 α -glucosidase inhibitory activity was expressed as percent inhibition and calculated same as α -glucosidase activity.

3.3.9 Caco-2 sucrase and glucoamylase activity

Sucrase and glucoamylase activity were determined following the procedure previously described by Oh et al. [16] with a slight modification. Differentiated Caco-2 cells were harvested with PBS. The Caco-2 cell lysate was then mixed with 200 mM of sucrose for testing sucrase inhibitory activity or 1% of starch for testing glucoamylase inhibitory activity. Degree of enzymatic activity was determined by amount of glucose released from substrate in a given period of time by using Invitrogen glucose peroxidation kit from Invitrogen. The absorbance was measured at 560 nm by a Micro-Plate Reader and compared with control. Caco-2 sucrase and glucoamylase activity

expressed as percent inhibition and calculated same as α -glucosidase activity.

3.3.10 qPCR

The mRNA expression of Caco-2 cells was evaluated by RT-PCR. Total RNAs from differentiated Caco-2 cells were isolated by using TRIzol reagent by following the manufacture instruction. The RNA pellet was dissolved in RNase-free water, and RNA concentration and purity were measured by using nanodrop machine (Fisher, Bridgewater, NJ, USA) prior to cDNA synthesis. 1 μ g of RNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA). Quantitative real time PCR reaction was performed with 100 ng of cDNA and 0.5 μ M of forward and reverse primer with 2X Fast SYBR green Master Mix under 40 cycles. The expression level of each target gene is normalized to GAPDH and calculation was followed by delta delta ct calculation method ($2^{\Delta\Delta ct}$). Primers for sodium glucose transporter 1 (SGLT1, forward (5'- GGG CAG CTT CAG GCA TC -3') and reverse (5'- AAA CAG CCA GCC CAG CA -3')), glucose transporter 2 (GLUT2, forward (5'- ACG GCT GGT ATC AGC AAA C -3') and reverse (5'- CTG CCT TCT CCA CAA GGA ATA C -3')), glucose transporter 5 (GLUT5, forward (5'- GGC TGG GAC TCT GTG GA -3') and reverse (5'- CAG CTT CAG CAC GGA GAT G -3')), sucrose-isomaltase (S-I, forward (5'- CCG GTG GCA TTC TGG ATT T -3') and reverse (5'- TAT GCT GGC ATT GCT GGT AG -3')), maltase-glucoamylase (M-G, forward (5'- TGC CTT CAC CAG TGT TT -3') and reverse (5'- GGG CAC TTC AAA CCT GTT ATT G -3')), TAS1R2 (T1R2, forward (5'- GCT GCC CAC CAA CTA CCA -3') and reverse (5'- CGC TGT AGG CAG ACA TAG A -3')), TAS1R3 (T1R3, forward (5'- CTG CTT TGG CCT GGT GTG -3') and reverse (5'- GTG GGA CCA GGG CTG CT

-3')), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, forward (5'- GAT GCT GGC GCT GAG TA -3') and reverse (5'- GGC AGC GAT GAT GAC CCT TT -3')) were used.

3.3.11 Glucose uptake

Differentiated Caco-2 cells were pre-incubated with serum free DMEM for 3 hrs. Medium was then replaced with serum-free high glucose (25 mM) with or without a sample for 1 hour. After an hour, media was replaced with 6-NBDG for 15 mins. Cells lysate was prepared with lysis buffer (0.1M potassium phosphate buffer (pH10) containing 1% Triton X-100) and transferred into 96-well plate. The fluorescence was immediately measured using micro-plate reader (Excitation=460, emission=540). The rate of glucose uptake was normalized to control.

3.3.12 Statistical Analysis

All data were presented as the mean \pm SD for each assay. Statistical analysis was carried out using SAS program (University edition SAS Inc. NC. USA) and significance of each group was verified with One-way analysis of variance (ANOVA) followed by the Duncan's multiple range test and the Student's t-test for comparison of means. p-values of less than 0.05 were considered to be statistically significant.

3.4 Results

3.4.1 Total phenolic contents and antioxidant capacity of five berry extracts

Total phenolic contents and antioxidant capacities of five different berries extracts, blackberry (BBE), cranberry (CBE), strawberry (SBE), raspberry (RBE), and blueberry (BLBE) extracts were evaluated to correlate the amount of phenolic contents with antioxidant capacity. As shown in Table 1, the amount of total phenolic contents among berry extracts varied from 28.55 ± 0.06 to 56.15 ± 1.08 gallic acid equivalent (GAE ug/mL). BBE showed the highest phenolic contents (28.55 ± 0.06 GAE ug/mL), whereas BLBE (28.55 ± 0.06 GAE ug/mL) showed the lowest total phenolic contents.

In order to determine antioxidant capacity of five different berry extracts, three different antioxidant assays, ferric reducing ability power, DPPH radical, and nitric oxide scavenging activity were performed as shown in Table 1. Ferric reducing ability power (FRAP) of berry extracts calculated using ascorbic acid equivalent (AAE μ M/mL). Among the five berries, BBE displayed the highest FRAP values (715 ± 20 AAE μ M), and BLBE has the lowest FRAP values (287 ± 9 AAE μ M). Antioxidant capacity of berry extracts against DPPH radicals (2,2-diphenyl-1-picrylhydrazyl) was quantified and half maximal inhibitory concentration (IC_{50}) was measured. Within five berry extracts, BBE showed the lowest concentration IC_{50} (1.15 ± 0.01 mg/mL) which implicated the highest DPPH scavenging activities. In contrast, CBE and BLBE showed the lowest DPPH radical scavenging activities ($IC_{50} > 2.5$ mg/mL). Antioxidant capacities of berry extracts against nitric oxide (NO) was also quantified and IC_{50} values were measured. Similar to other antioxidant assays, BBE had the highest efficacy for scavenging nitric oxide (IC_{50} : 2.46 ± 0.08 mg/mL), whereas CBE and BBE showed the lowest activity (IC_{50} : > 5 mg/mL) for scavenging nitric oxide (Table 1). These results

indicate that antioxidant capacity is strongly correlated with total phenolic contents

Total Phenolic Contents & Antioxidant Activities of Berry extracts				
Equivalent value of berry antioxidants				
	TPC	FRAP	DPPH	NO
	GAE (µg/mL)	AAE (µM/mL)	IC ₅₀ (mg/mL)	IC ₅₀ (mg/mL)
BBE	56.15 ± 1.08 ^a	715 ± 20 ^a	1.15 ± 0.01	2.46 ± 0.08
SBE	54.90 ± 1.23 ^a	582 ± 16 ^b	1.22 ± 0.08	2.98 ± 0.16
RBE	34.65 ± 0.63 ^b	450 ± 36 ^c	1.95 ± 0.16	4.13 ± 0.03
CBE	33.28 ± 0.57 ^b	339 ± 26 ^d	> 2.5	> 5
BLBE	28.55 ± 0.06 ^c	287 ± 9 ^e	> 2.5	> 5

Table 1. Total phenolic contents and antioxidant capacity of five different berries.

Results were expressed as mean ± SD of three independent experiments in triplicate. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. TPC: total phenolic contents, FRAP: Ferric reducing ability of plasma, DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, NO: Nitric oxide inhibitory activity, GAE: Gallic acid equivalent, AAE: Ascorbic acid equivalent, BBE: Blackberry extracts, SBE: Strawberry extracts, RBE: Raspberry extracts, CBE: Cranberry extracts, BLBE: Blueberry extracts.

3.4.2 Effect of different berry extract on rat and Caco-2 α -glucosidase activity

The effects of different berry extracts on rat α -glucosidase inhibitory activities was evaluated for screening antidiabetic potential of berry extracts, and 25 μ M of Acarbose was included as a positive control. As shown in Figure 1 (A), all berry extracts dose dependently increased rat α -glucosidase inhibitory activity. However, CBE and BBE showed the highest rat α -glucosidase inhibitory activity by 74% and 72% respectively, while other berry extracts inhibited the rat α -glucosidase activity by around 65% at 5 mg/mL concentration.

Many published studies have investigated antidiabetic effects of various berry extracts by determining yeast [28] and rat α -glucosidase [29], which is often difficult to extrapolate the activity of data to humans. Therefore, effects of berry extracts on human α -glucosidase activity was evaluated by using human Caco-2 intestinal cells. As shown in Figure 1 (B), effect of berry extracts on Caco-2 α -glucosidase activity showed similar trends as rat α -glucosidase activity (Data was not included 5 mg/mL concentration due to all extracts reached 100% inhibition). CBE showed the highest human Caco-2 α -glucosidase inhibitory activity (95%) at 2.5 mg/mL concentration, followed by black, and other three berry extracts. However, berry extracts showed higher inhibitory effects in human Caco-2 cells rather than rat α -glucosidase.

So far, the correlation between total phenolic contents and α -glucosidase activity remains controversial, however, the amount of phenolic content are believed to correlate with α -glucosidase inhibitory activities [127]. However, in our study, amount of total phenolic contents does not seem to correlate with α -glucosidase activity.

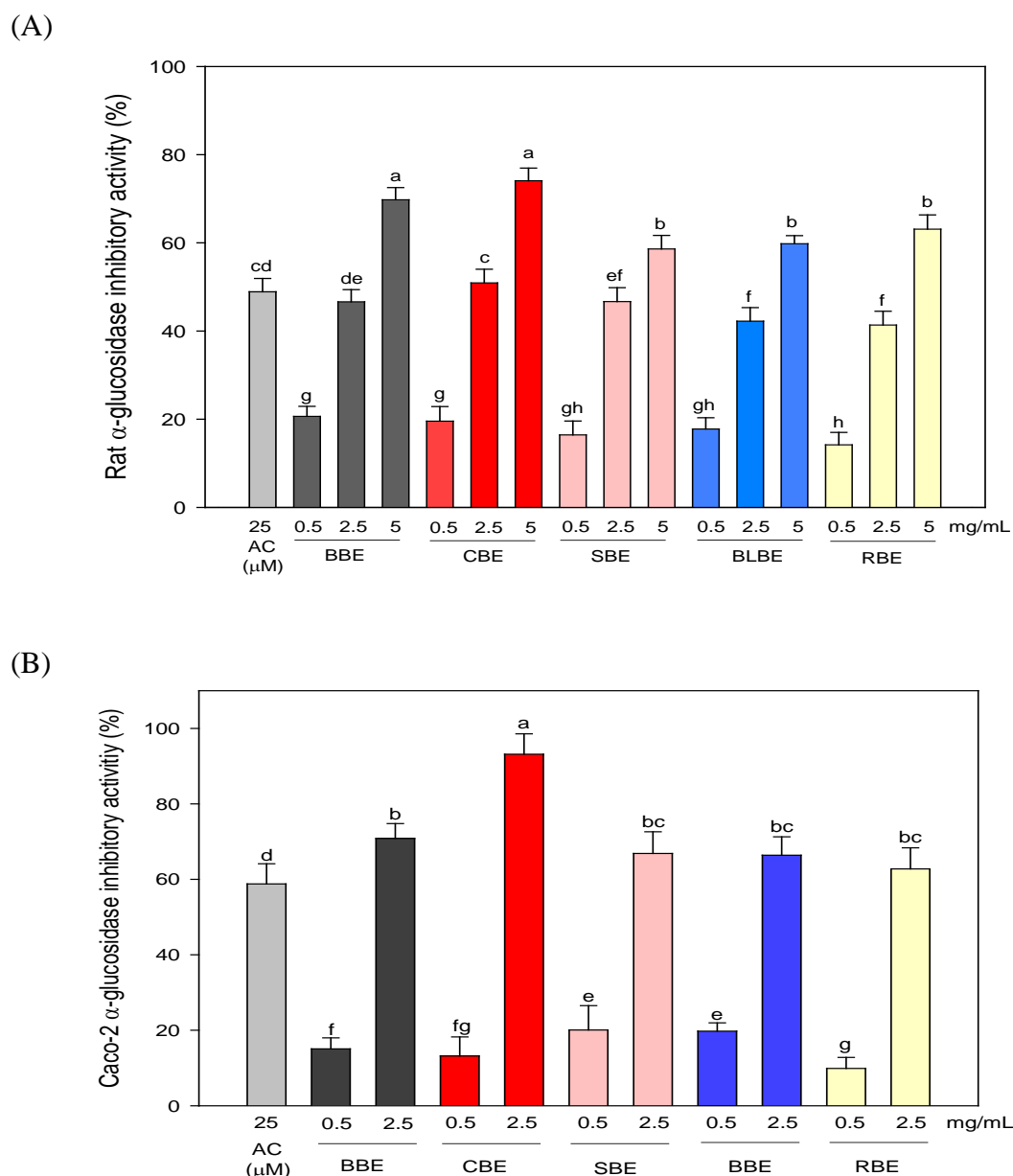


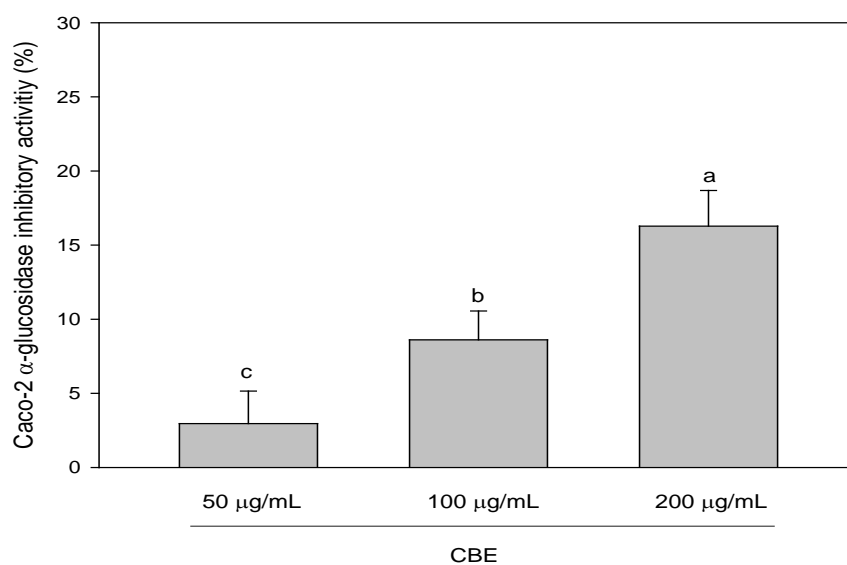
Figure 1. Effects of five different berry extracts on rat and Caco-2 α -glucosidase activity. Rat and Caco-2 α -glucosidase activity were determined by measuring p-nitrophenol released from pNPG at 405 nm. Results are expressed as mean \pm SD of three independent experiments each performed in triplicates. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. AC: Acarbose, BBE: Blackberry extracts, SBE: Strawberry extracts, RBE: Raspberry extracts, CBE: Cranberry extracts, BLBE: Blueberry extracts.

3.4.3 Effects of CBE and BBE on sucrase and glucoamylase activity

Among five different berries, we selected CBE and BBE based on the highest α -glucosidase inhibitory activity and total phenolic contents. However, in order to evaluate the α -glucosidase inhibitory effects of berry extracts at physiological concentration, we evaluated effects of CBE and BBE on Caco-2 α -glucosidase activity at low concentration (0 ~ 200 $\mu\text{g/mL}$). As shown in Figure 2, both CBE and BBE inhibited Caco-2 α -glucosidase activities in dose dependent manner.

α -glucosidase is a cluster of several enzymes in the small intestine. Among the α -glucosidases, retardation of sucrase and glucoamylase activity are directly involved in postprandial hyperglycemia due to sucrose and starch are commonly consumed from our diet. Therefore, we evaluated effects of berry extracts on sucrase and glucoamylase activity. As shown in Figure 3, both CBE and BBE suppressed sucrase activities even at 50 $\mu\text{g/mL}$ concentration and sucrase inhibitory activity were increased dose dependently. Although the both CBE and BBE showed inhibitory effects of glucoamylase at 200 $\mu\text{g/mL}$ concentration, only CBE seems to have glucoamylase inhibitory effects at 50 to 100 $\mu\text{g/mL}$ concentrations (Figure 4). These data suggest that CBE and BBE have antidiabetic potentials by inhibiting sucrase and glucoamylase, but the efficacy of berry extracts on specific enzyme inhibitory effects may differ.

(A)



(B)

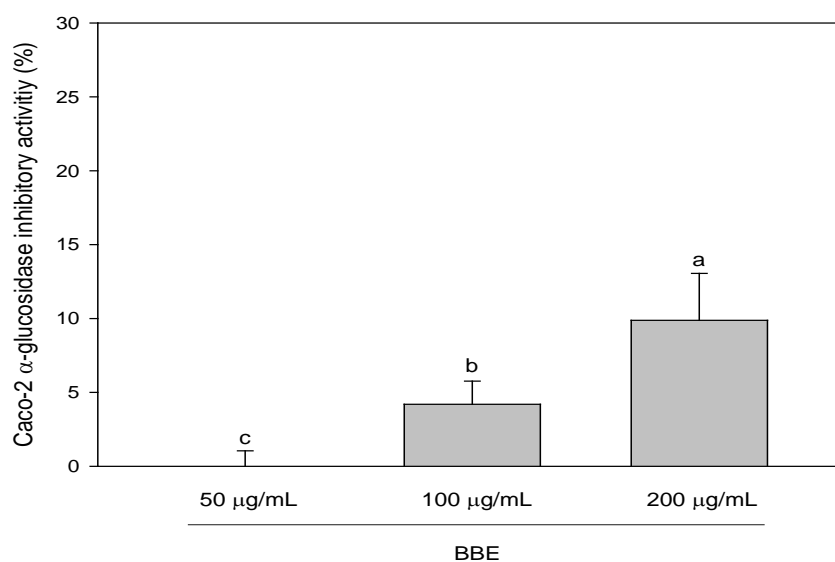
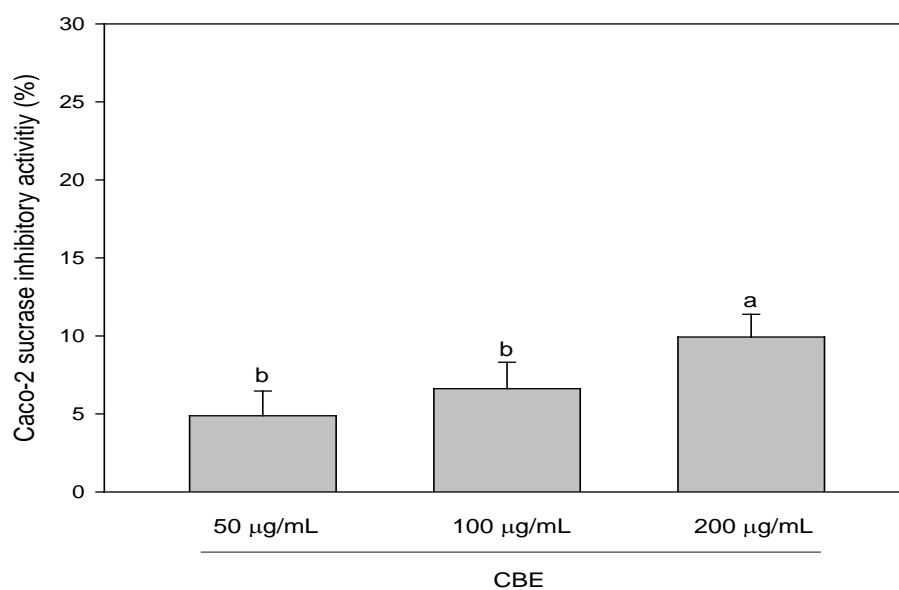


Figure 2. Effects of CBE and BBE on Caoc-2 α -glucosidase activity. Caco-2 α -glucosidase activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. Results are expressed as mean \pm SD of three independent experiments each performed triplicate. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts.

(A)



(B)

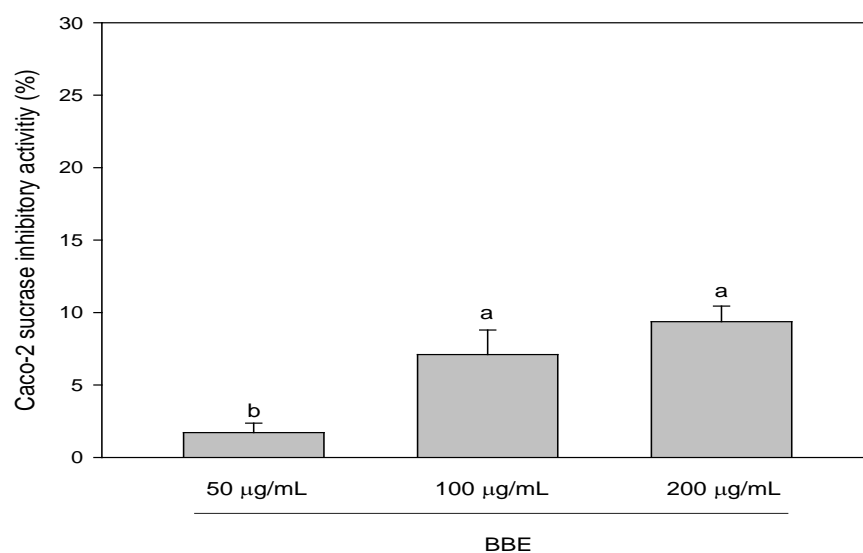
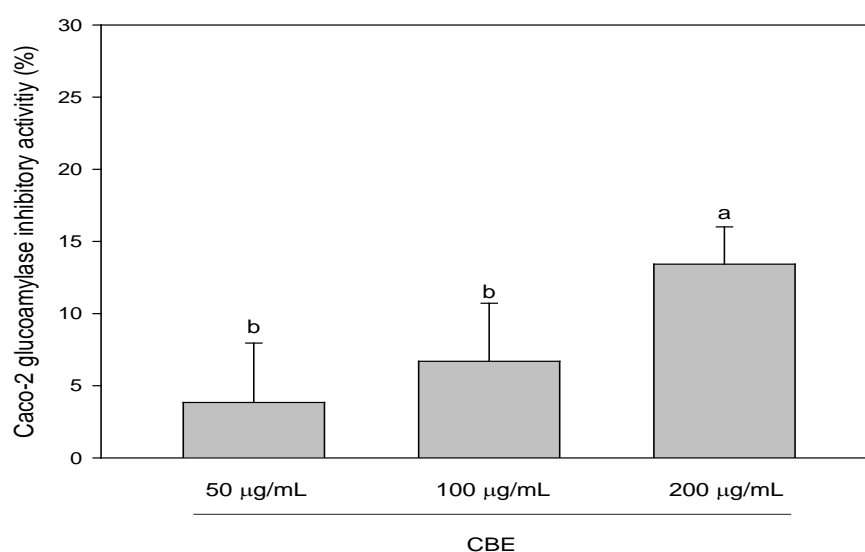


Figure 3. Effects of CBE and BBE on Caco-2 sucrase activity. Sucrase inhibitory activity was determined by measuring glucose released from sucrose at 560 nm by using glucose peroxidation kit. Results are expressed as mean \pm SD of three independent experiments each performed triplicate. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts.

(A)



(B)

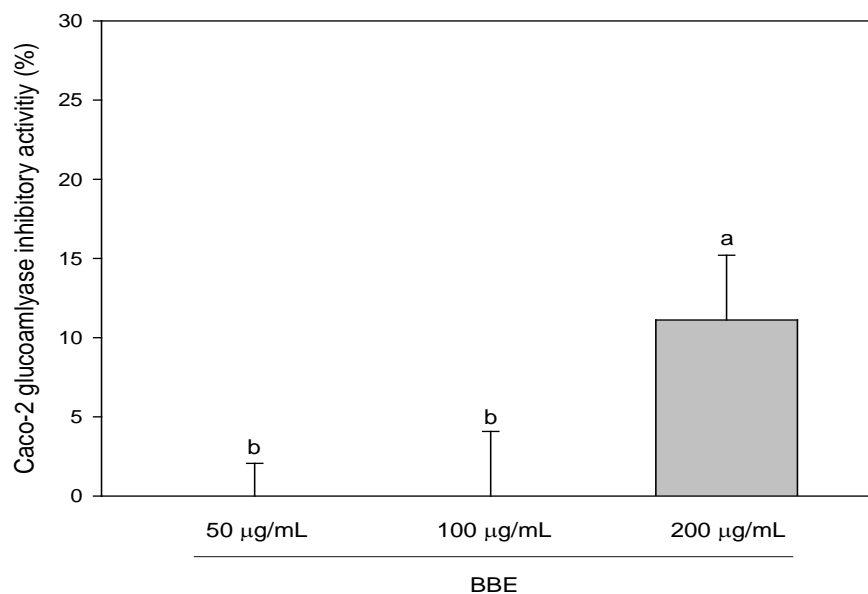


Figure 4. Effects of CBE and BBE on Caco-2 glucoamylase activity. Glucoamylase inhibitory activity was determined by measuring glucose released from starch at 560 nm by using glucose peroxidation kit. Results are expressed as mean \pm SD of three independent experiments each performed triplicate. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts

3.4.4 Effects of CBE and BBE on glucose uptake and gene expression in Caco-2 cells

Berry mixtures have been reported to suppress the glucose uptake by inhibiting glucose transporters [30], but individual berries were not examined. Individual berry extracts may have different molecular mechanisms to modify postprandial plasma glucose level. Hence, we investigated effects of CBE and BBE on glucose uptake as well as gene expression of glucose transporters and carbohydrate digesting enzymes in the small intestine.

Physiological effects of CBE and BBE on enterocytes were determined by glucose uptake using 6-NBDG fluorescence probe. As shown in Figure 5, both CBE and BBE significantly decreased glucose uptake at 200 $\mu\text{g/mL}$ concentration. However, only CBE reduced glucose uptake even at 100 $\mu\text{g/mL}$ concentration.

In order to identify molecular mechanisms of CBE and BBE, glucose transporters, SGLT1 and GLUT2, and two subunits of α -glucosidase, S-I and M-G, were evaluated. As shown in Figure 6, both glucose transporters were significantly decreased in the presence of CBE at 200 $\mu\text{g/mL}$. Also, both carbohydrate digesting enzymes were significantly decreased at 200 $\mu\text{g/mL}$ (Figure 7). On the other hand, BBE did not alter the both SGLT1 and GLUT2 mRNA expression, although BBE significantly reduced expression of S-I and M-G at 200 $\mu\text{g/mL}$ concentration (Figure 10). These results suggest that CBE and BBE have different molecular mechanisms to prevent postprandial hyperglycemia.

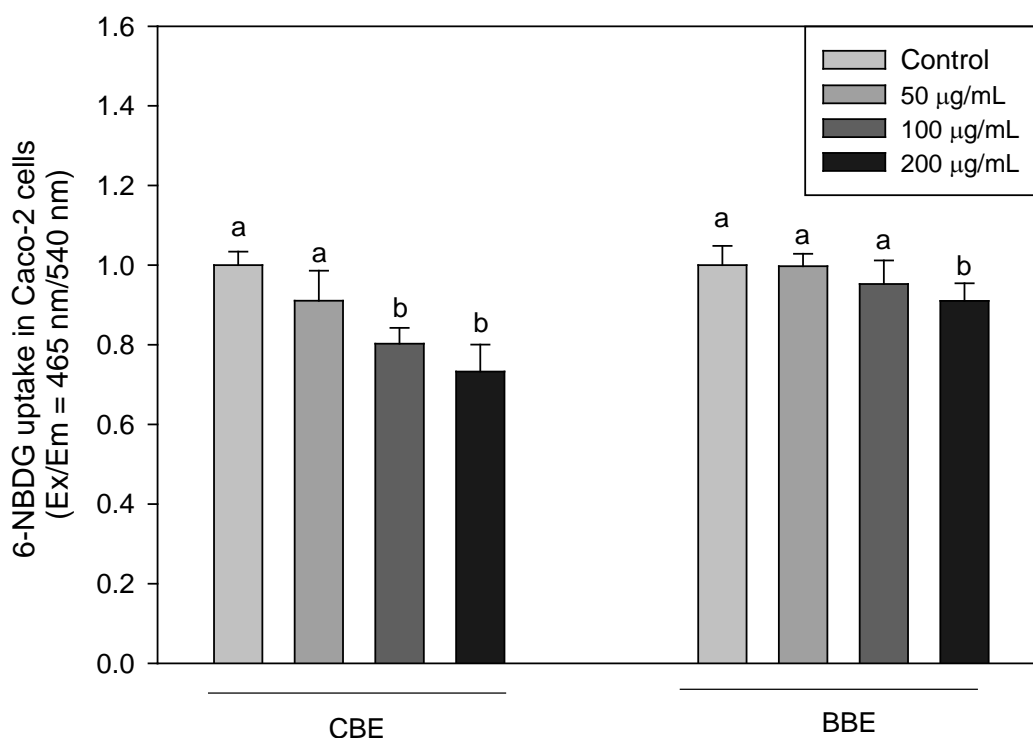


Figure 5. Effects of CBE and BBE on glucose uptake in human Caco-2 intestinal cells. Human intestinal Caco-2 cells were treated with CBE or BBE extracts for 1hr. Glucose uptake was determined by measuring fluorescence of 6-NBDG in the cells. Excitation and emission wavelength for 6-NBDG is 465 nm and 540 nm respectively. Results are normalized to control group and presented as mean \pm SD of three independent experiments (n=3). CBE: Cranberry extracts, BBE: Blackberry extracts.

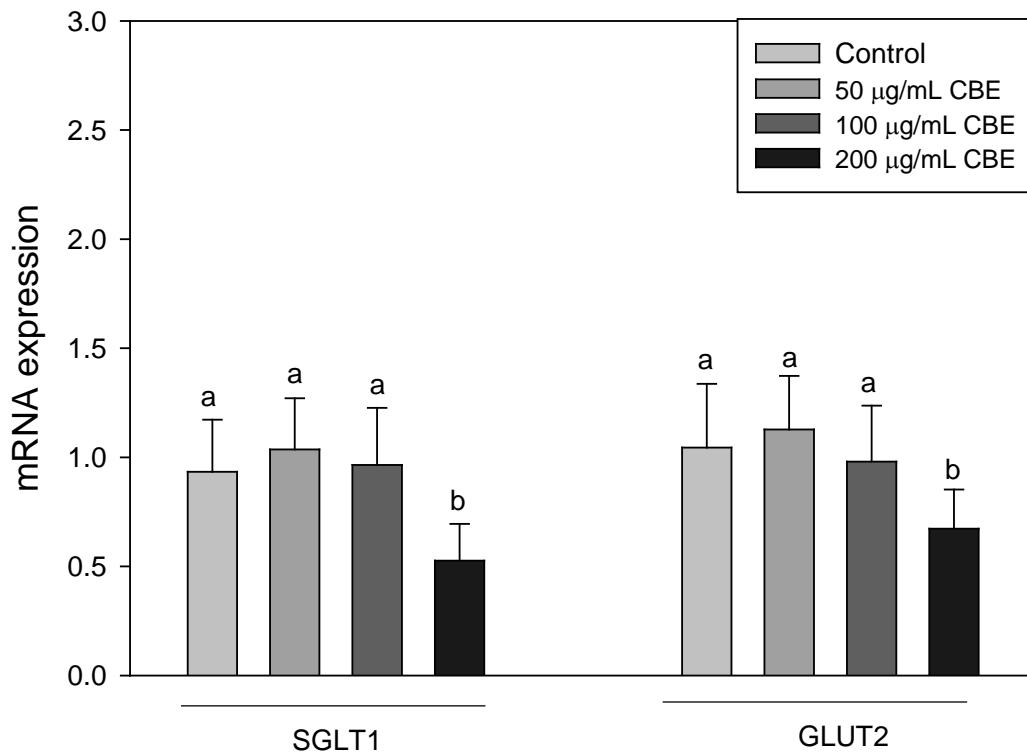


Figure 6. Effects of CBE on glucose transporter expression. Human intestinal Caco-2 cells were treated with different concentrations of CBE (0~200 µg/mL) for 1hr. SGLT1 and GLUT2 mRNA level were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts.

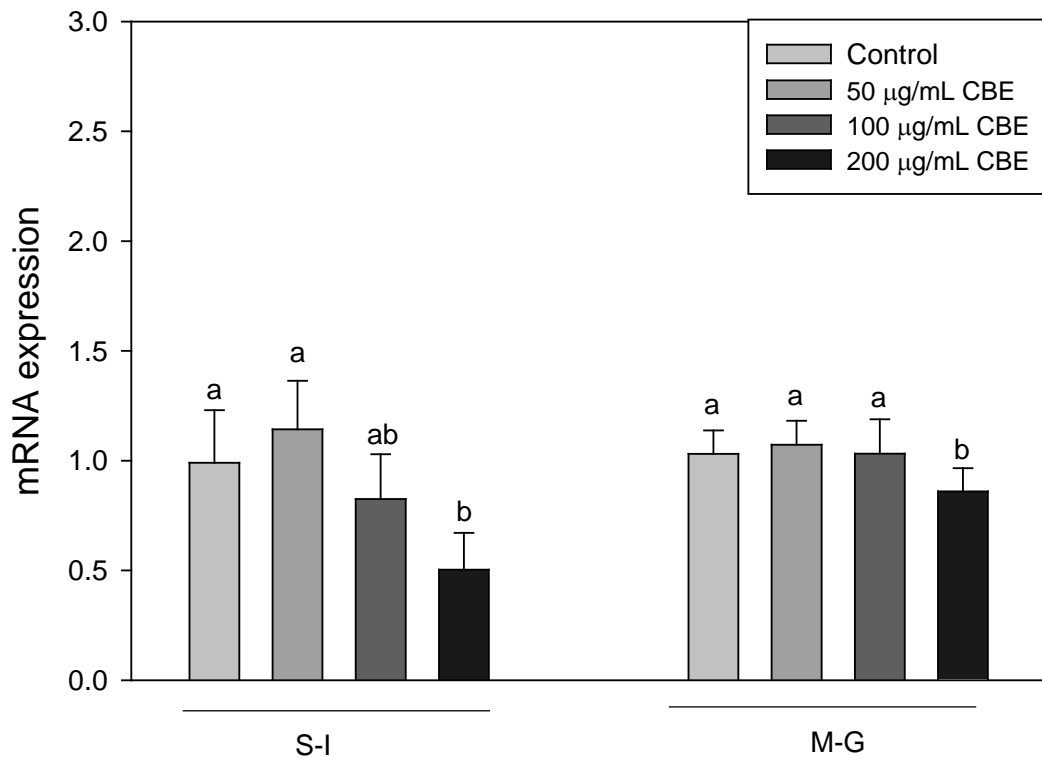


Figure 7. Effects of CBE on carbohydrate digesting enzyme expression. Human intestinal Caco-2 cells were treated with different concentrations of CBE (0~200 µg/mL) for 1hr. S-I and M-G mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts.

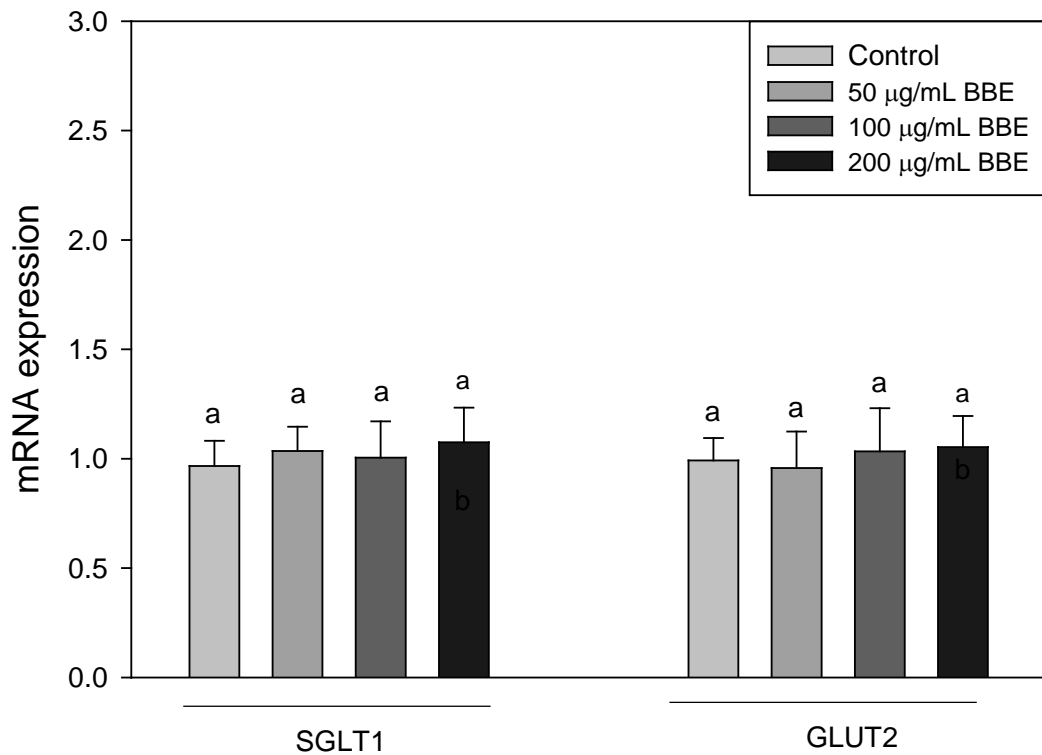


Figure 8. Effects of BBE on glucose transporter expression. Human intestinal Caco-2 cells were treated with different concentrations of BBE (0~200 µg/mL) for 1hr. SGLT1 and GLUT2 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. BBE: Blackberry extracts.

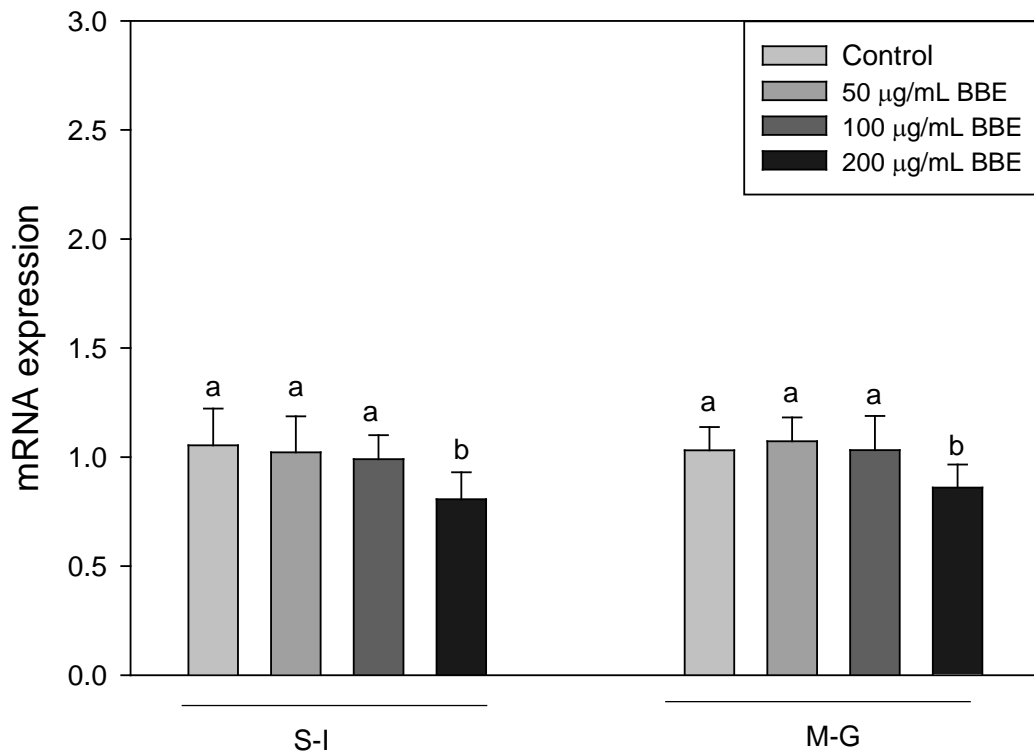


Figure 9. Effects of BBE on carbohydrate digesting enzyme expression. Human intestinal Caco-2 cells were treated with different concentrations of BBE (0~200 µg/mL) for 1hr. S-I and M-G mRNA level were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. BBE: Blackberry extracts.

3.4.5 Effects of sucrose on small intestinal α -glucosidase, glucose transporters, and glucose uptake in Caco-2 cells

Dietary sucrose has been considered as a major determinant of postprandial hyperglycemia. However, effects of sucrose on small intestinal α -glucosidase and glucose transporters were not well understood in *in vitro* setting. Therefore, we selected the concentrations ranging from 25 mM to 100 mM of sucrose to determine the effects of sucrose on intestinal digestion and absorption in the small intestine.

In order to determine physiological effects of sucrose on enterocytes, glucose uptake was performed by using 6-NDBG probe. As shown in Figure 10, there were no changes until 100 mM of sucrose. When the cells are exposed to 100 mM of sucrose, glucose uptake was significantly increased by 30%

Sucrose is hydrolyzed into glucose and fructose, which are then transported by glucose transporters (SGLT1, GLUT2) and fructose transporter (GLUT2, GLUT5) into enterocytes. As shown in Figure 11, mRNA expression of SGLT1, GLUT2, and GLUT5 was not changed in the presence of 50 mM of sucrose. However, mRNA expression of these glucose transporters was significantly elevated by around 20~30% at 100 mM of sucrose compare to control

From Between two subunits of α -glucosidase, only sucrose hydrolyzing enzyme, S-I, was significantly increased in presence of 100 mM sucrose, whereas starch hydrolyzing enzyme, M-G, was not altered in the presence of 100 mM of sucrose (Figure 12). Therefore, 100 mM sucrose were further tested for evaluating effects of CBE and BBE in presence of dietary sucrose.

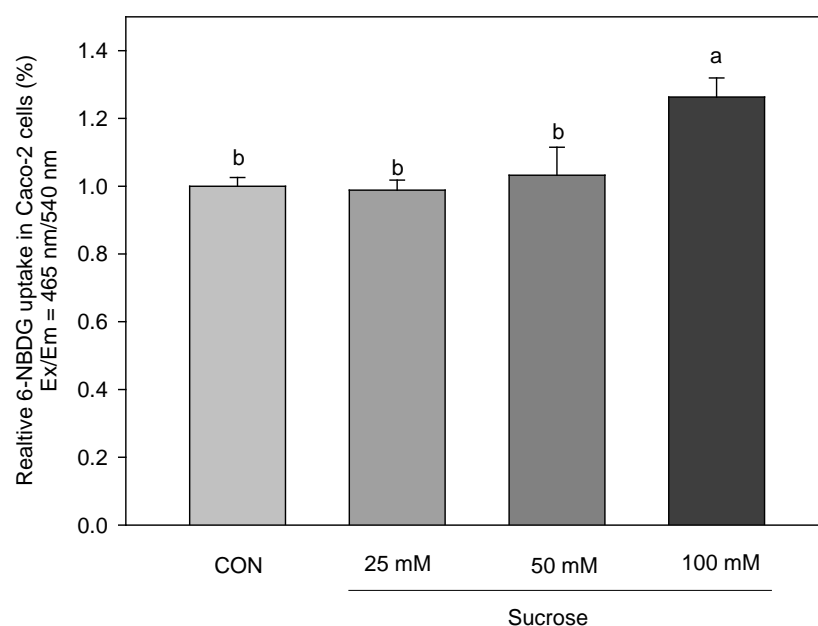


Figure 10. Effects of sucrose on glucose uptake. Human intestinal Caco-2 cells were treated with different concentration of sucrose for 1hr. Glucose uptake was determined by measuring fluorescence of 6-NBDG in the cells. Excitation and emission wavelength for 6-NBDG is 465 nm and 540 nm respectively. Results are normalized to control group and presented as mean \pm SD of three independent experiments (n=3).

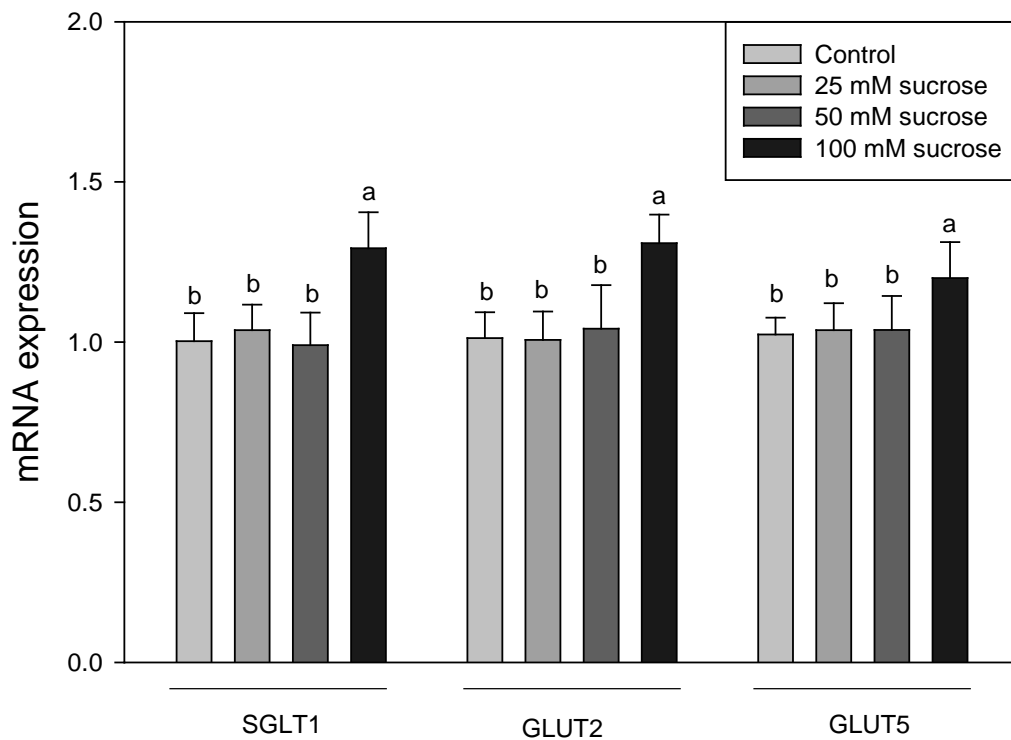


Figure 11. Effects of sucrose on glucose transporter expression. Human intestinal Caco-2 cells were treated with different concentrations of sucrose (25~100 mM) for 1hr. SGLT1, GLUT2, and GLUT5 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test.

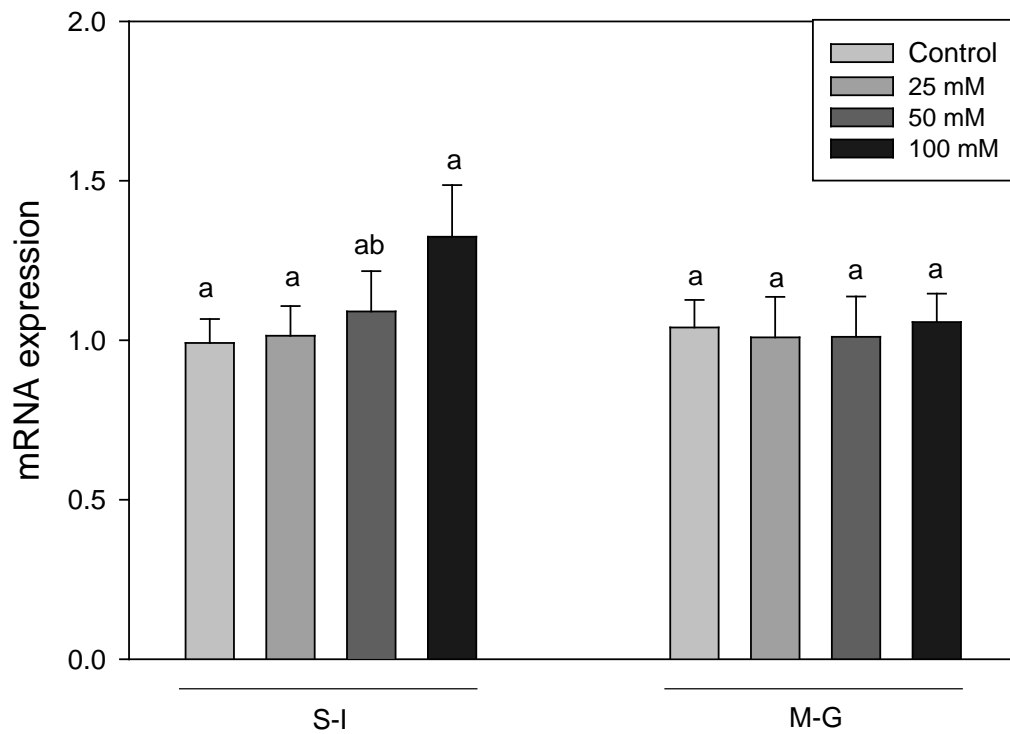


Figure 12. Effects of sucrose on carbohydrate digesting enzyme expression. Human intestinal Caco-2 cells were treated with different concentrations of sucrose (25 ~ 100 mM) for 1hr. S-I and M-G mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test.

3.4.6 Effects of CBE and BBE on small intestinal α -glucosidase, glucose transporters, and glucose uptake in Caco-2 cells

Next, we investigated the effects of CBE and BBE on intestinal α -glucosidase, glucose transporters as well as glucose uptake in the presence of sucrose. Physiological effects of CBE and BBE on human intestinal cells were determined by glucose uptake (Figure 13). In the presence of 100 mM of sucrose, glucose uptake was significantly increased, which was markedly decreased in the presence of CBE or BBE.

Effect of CBE and BBE on glucose transporters and α -glucosidase enzymes in the presence of sucrose were determined. As shown in Figure 14, mRNA levels of SGLT1, GLUT2, and GLUT5 were significantly increased by 100 mM sucrose, which was significantly attenuated in presence of CBE and BBE. Furthermore, S-I was elevated in the presence of 100 mM of sucrose and was diminished in the presence of CBE or BBE. In the case of the M-G, it was not altered in the presence of dietary sucrose but it was downregulated by the CBE or BBE (Figure 15). These results suggest that CBE and BBE may regulate postprandial hyperglycemia in high sucrose diet by modifying glucose transporters as well as carbohydrate digesting enzymes.

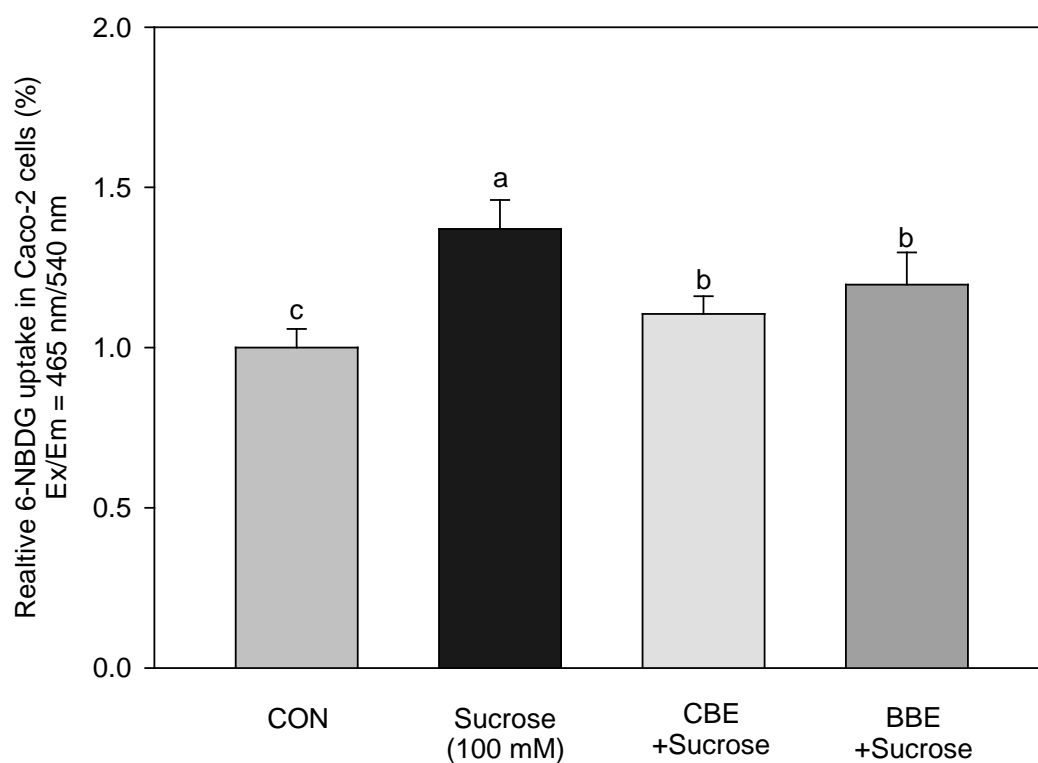


Figure 13. Effects of CBE and BBE on glucose uptake in the presence of sucrose.

Human intestinal Caco-2 intestinal cells were treated with 200 µg/mL of CBE or BBE extracts in the presence of 100 mM of sucrose for 1hr. Glucose uptake was determined by measuring fluorescence of 6-NBDG in the cells. Excitation and emission wavelength for 6-NBDG is 465 nm and 540 nm respectively. Results are normalized to control group and presented as mean \pm SD of three independent experiments (n=3). CBE: Cranberry extracts, BBE: Blackberry extracts

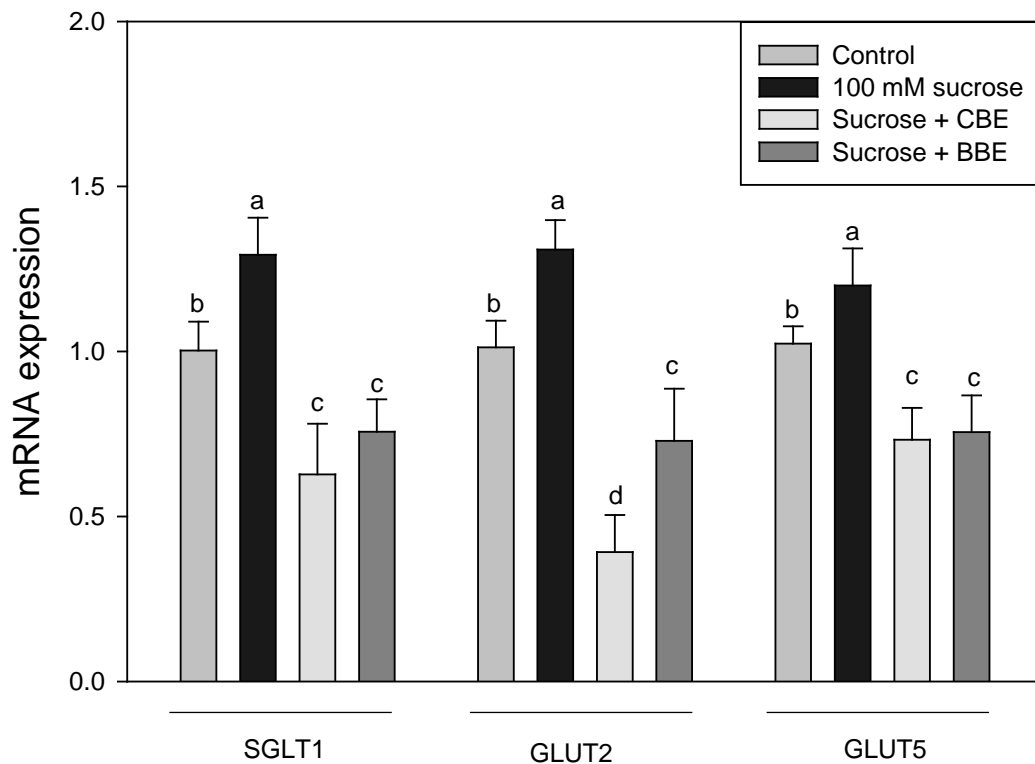


Figure 14. Effects of CBE and BBE on glucose transporter expression in the presence of sucrose. Human intestinal Caco-2 intestinal cells were treated with 200 $\mu\text{g/mL}$ of CBE or BBE in the presence of sucrose (100 mM) for 1hr. SGLT1, GLUT2, and GLUT5 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts. CBE: Cranberry extracts, BBE: Blackberry extracts

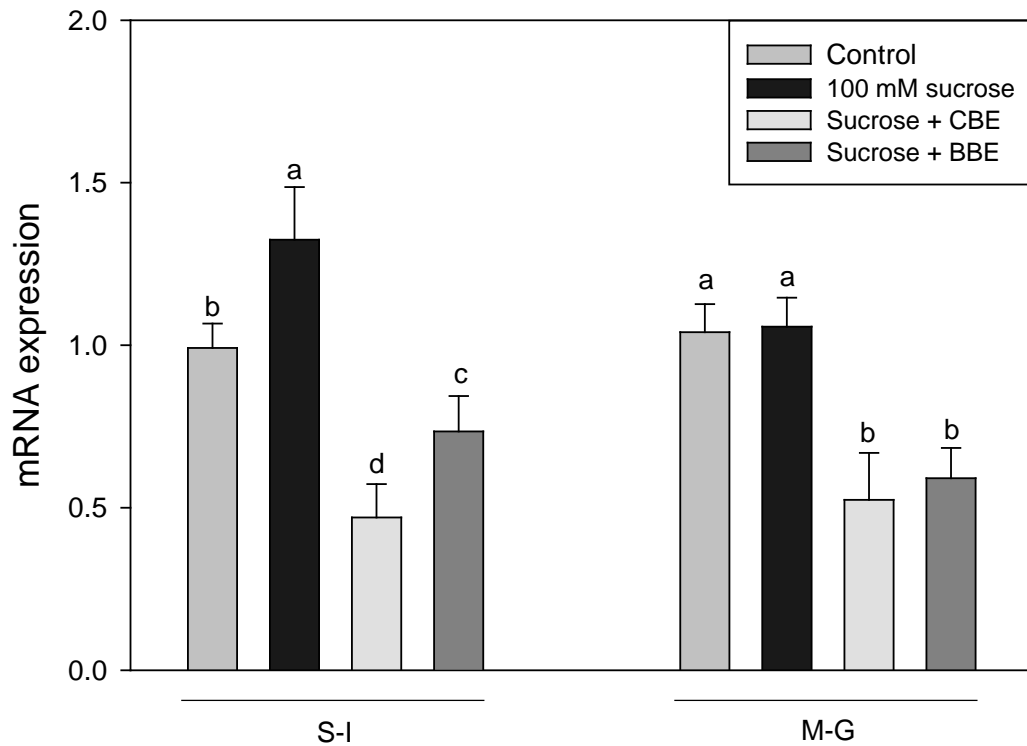


Figure 15. Effects of sucrose on carbohydrate digesting enzyme expression in the presence of sucrose. Human intestinal Caco-2 intestinal cells were treated with 200 $\mu\text{g/mL}$ of CBE or BBE in the presence of sucrose (100 mM) for 1hr. S-I and M-G mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts. CBE: Cranberry extracts, BBE: Blackberry extracts

3.4.7 Effects of sucralose on small intestinal α -glucosidase, glucose transporters, sweet-sensing receptors and glucose uptake in Caco-2 cells

Sucralose is one of artificial sweeteners that have been extensively used in various food products and beverages as an alternative to dietary sucrose [9]. Although sucralose is non-digestible and non-absorbable, there is evidence showing that dietary AS increased intestinal glucose transporters [12]. However, effects of sucralose on intestinal digestion and absorption are not currently known. Hence, the effect of sucralose was evaluated with different concentrations selected based on daily accepted intake of sucralose from FDA.

Glucose uptake was evaluated to identify physiological effects of sucralose on glucose absorption in enterocytes. As shown in Figure 16, glucose uptake was not increased in the presence of 1 mM sucralose but it was significantly elevated at 10 mM of sucralose compared to control.

As shown in Figure 17, 10 mM of sucralose significantly elevated SGLT1 and GLUT2 expression significantly without altering GLUT5. Effects of sucralose on α -glucosidase activity and its mRNA expressions were evaluated. Unlike sucrose, both α -glucosidase activity and gene expression of S-I and M-G, was not altered in the presence of sucralose (Figure 18). Expression of both sweet sensing receptors, T1R2 and T1R3 in Caco-2 cells were also evaluated and were increased dose dependently (Figure 19). Although molecular mechanisms between sweet-sensing receptors and SGLT1 are yet to be identified, the expression of T1R2 and T1R3 mRNA was similarly increasing as SGLT1 by sucralose treatment.

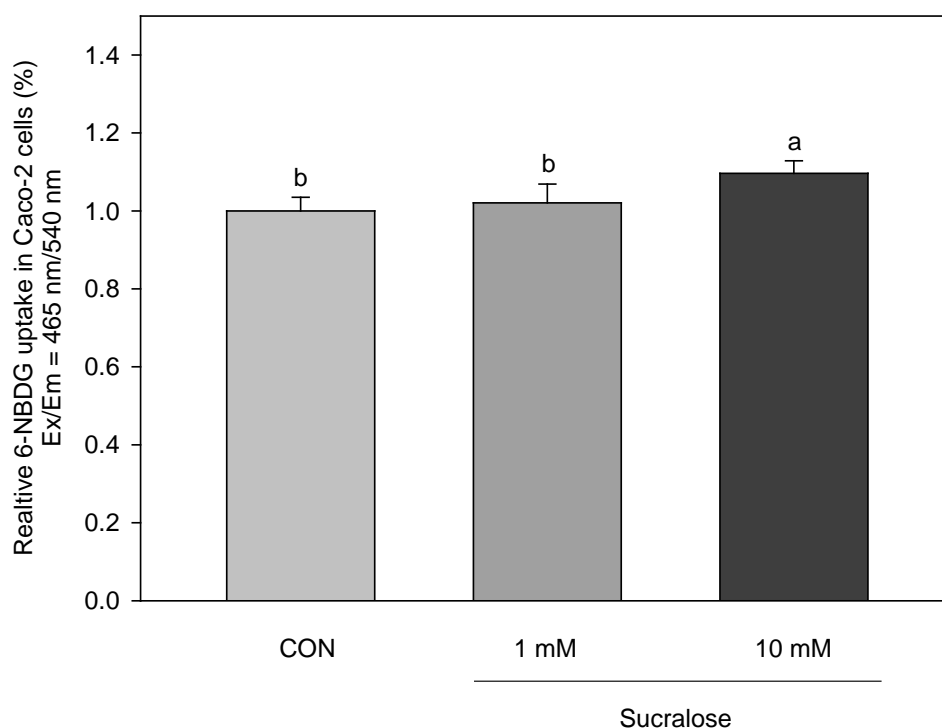


Figure 16. Effects of sucralose on glucose uptake human Caco-2 intestinal cells were treated with different concentration of sucralose for 1hr. Glucose uptake was determined by measuring fluorescence of 6-NBDG in the cells. Excitation and emission wavelength for 6-NBDG is 465 nm and 540 nm respectively. Results are normalized to control group and presented as mean \pm SD of three independent experiments (n=3).

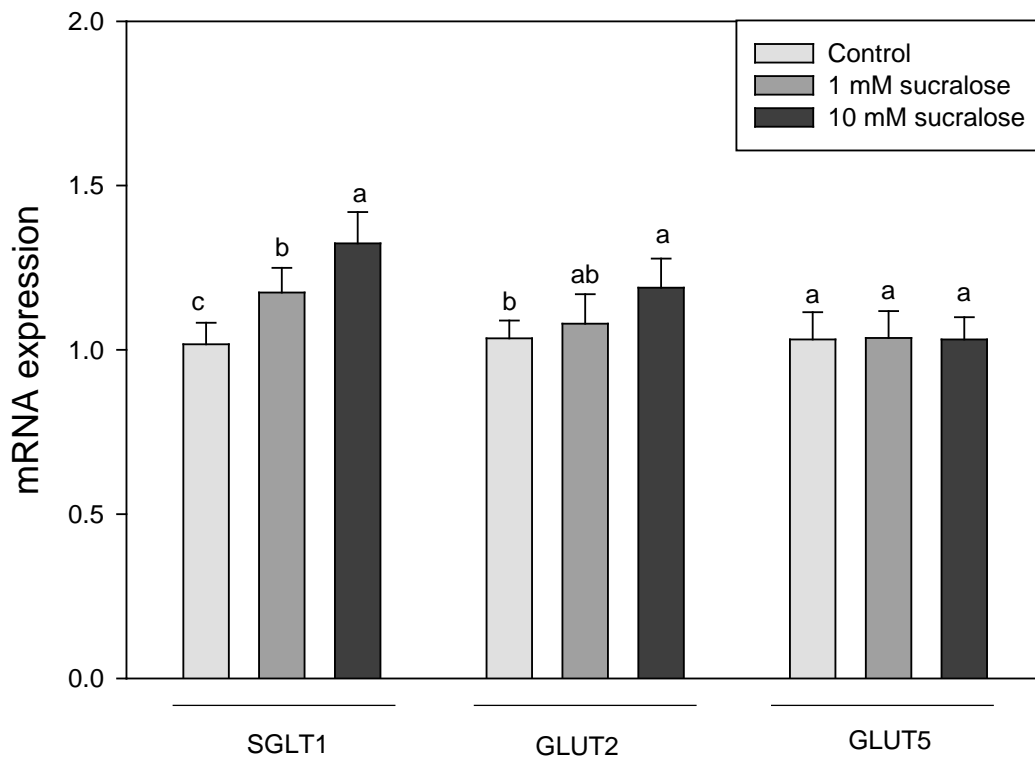


Figure 17. Effects of sucralose on glucose transporter expression. human Caco-2 intestinal cells were treated with different concentrations of sucralose (1, 10 mM) for 1hr. SGLT1, GLUT2, and GLUT5 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test.

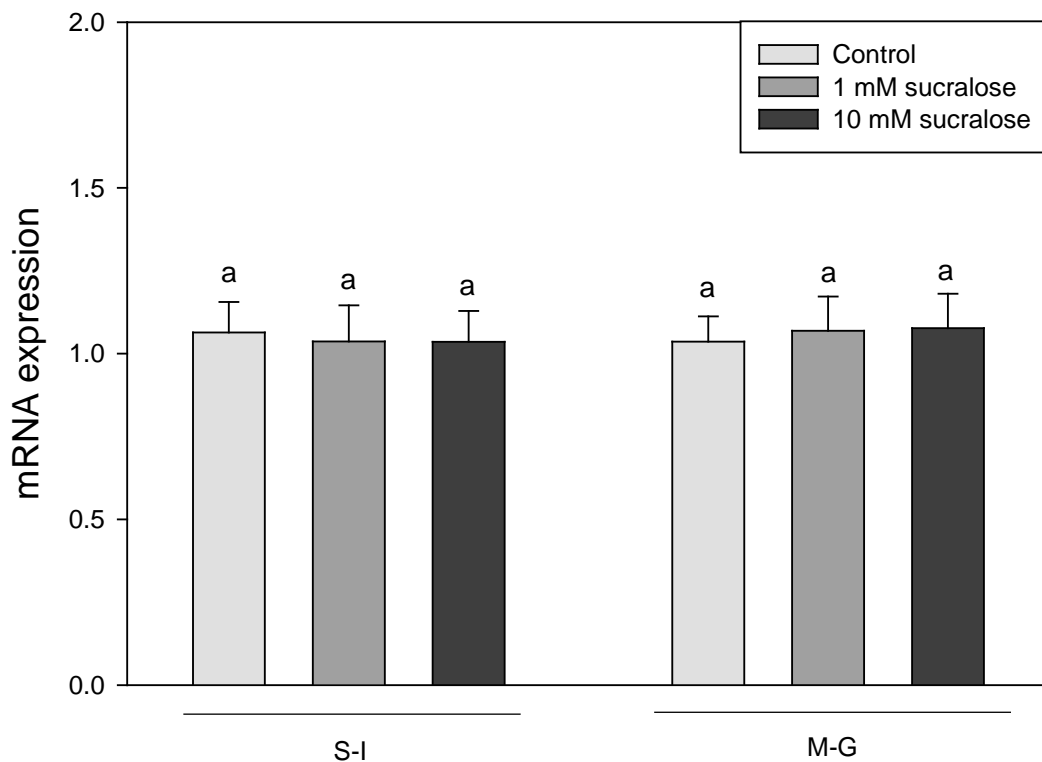


Figure 18. Effects of sucralose on carbohydrate digesting enzyme expression.

Caco-2 cells were treated with different concentrations of sucralose (1, 10 mM) for 1hr. S-I and M-G mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD with three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test.

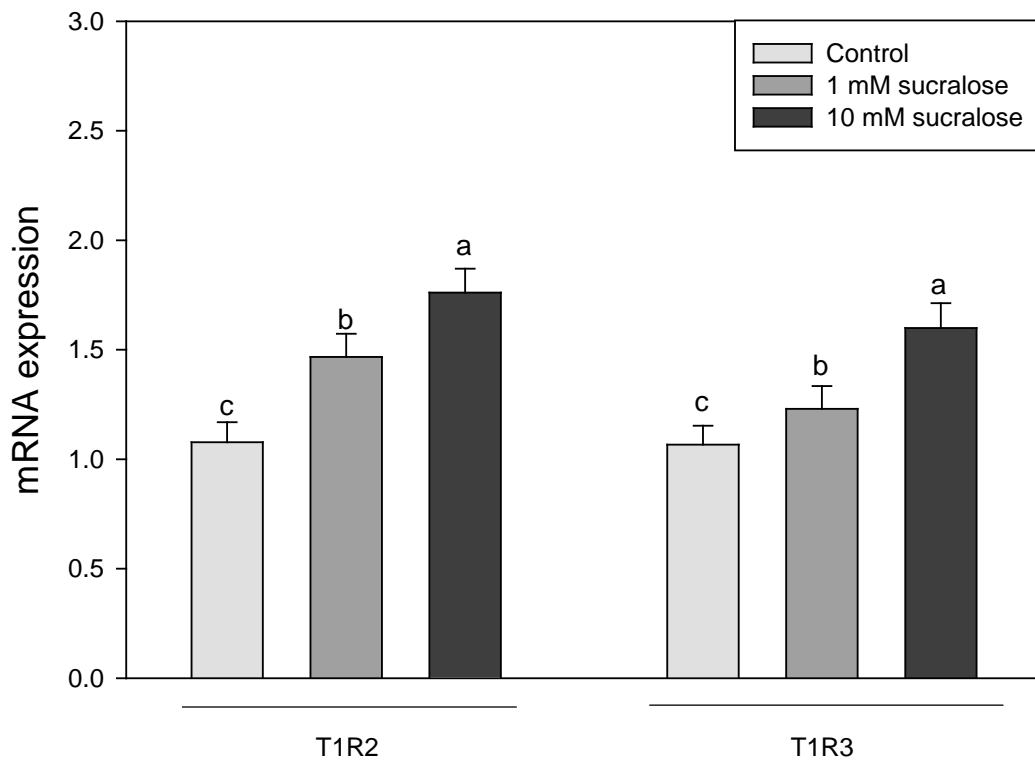


Figure 19. Effects of sucralose on sweet-sensing receptors expression. human Caco-2 intestinal cells were treated with different concentrations of sucralose (1, 10 mM) for 1hr. T1R2 and T1R3 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts.

3.4.8 Effects of CBE and BBE on small intestinal α -glucosidase, glucose transporters, sweet-sensing receptors and glucose uptake in the presence of sucralose in Caco-2 cells

To determine physiological effects CBE or BBE on enterocytes in the presence of 10 mM of sucralose, glucose uptake was performed (Figure 20). Compared to control, 10 mM sucralose elevated glucose uptake by 10%, but suppressed in the presence of 200 μ g/mL of CBE or BBE.

Next, we evaluated the molecular mechanisms of CBE and BBE on intestinal digestive enzymes and sweet-sensing receptors in the presence of 10 mM of sucralose. As shown in Figure 22, BBE suppressed all glucose transporters, SGLT1, GLUT2, and GLUT5, whereas CBE only suppressed GLUT2 and GLUT 5 without effect on the SGLT1 expression in presence of 10 mM of sucralose. Furthermore, sweet-sensing receptors, T1R2 and T1R3, were elevated in presence of sucralose and were attenuated in the presence of 200 μ g/mL of CBE or BBE (Figure 23). Moreover, between CBE and BBE, BBE had higher inhibitory effects on the T1R2 expression than CBE. As shown in previous results, sucralose did not affect the expression of carbohydrate- digesting enzymes. However, CBE and BBE suppressed S-I, and M-G mRNA expression (Figure 22).

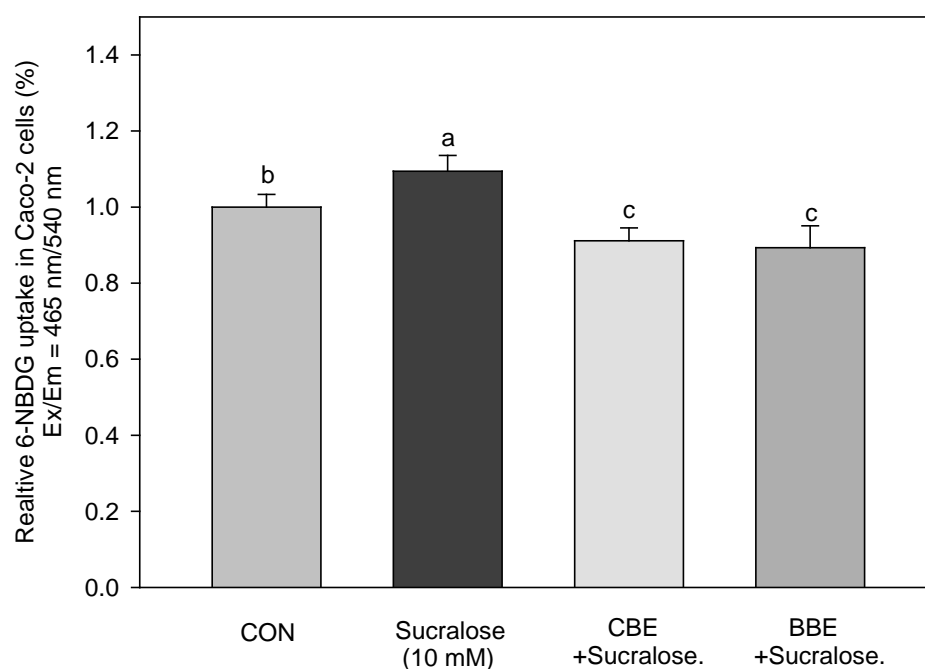


Figure 20. Effects of CBE and BBE on glucose uptake in the presence of sucralose.

Human intestinal Caco-2 cells were treated with 200 µg/mL of CBE or BBE extracts in presence of 10 mM of sucralose for 1hr. Glucose uptake was determined by measuring fluorescence of 6-NBDG in the cells. Excitation and emission wavelength for 6-NBDG is 465 nm and 540 nm respectively. Results are normalized to control group and presented as mean \pm SD of three independent experiments (n=3). CBE: Cranberry extracts, BBE: Blackberry extracts.

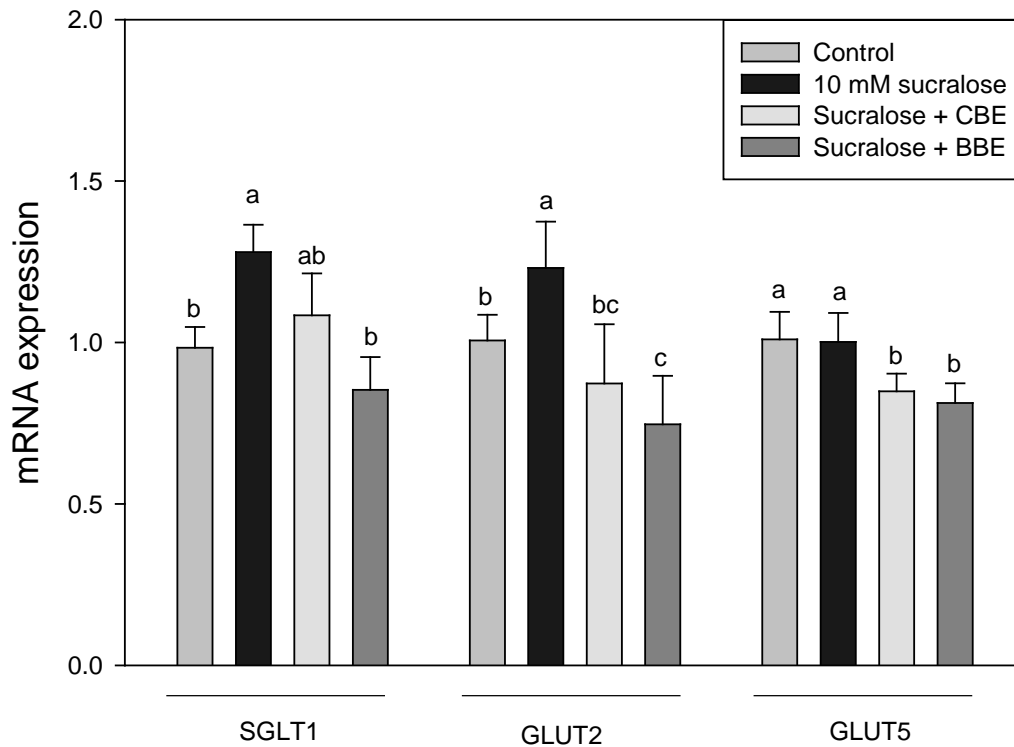


Figure 21. Effects of CBE and BBE on glucose transporter expression in the presence of sucralose Human intestinal Caco-2 were treated with 200 μ g/mL of CBE or BBE in presence of sucralose (10 mM) for 1hr. SGLT1, GLUT2, and GLUT5 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts.

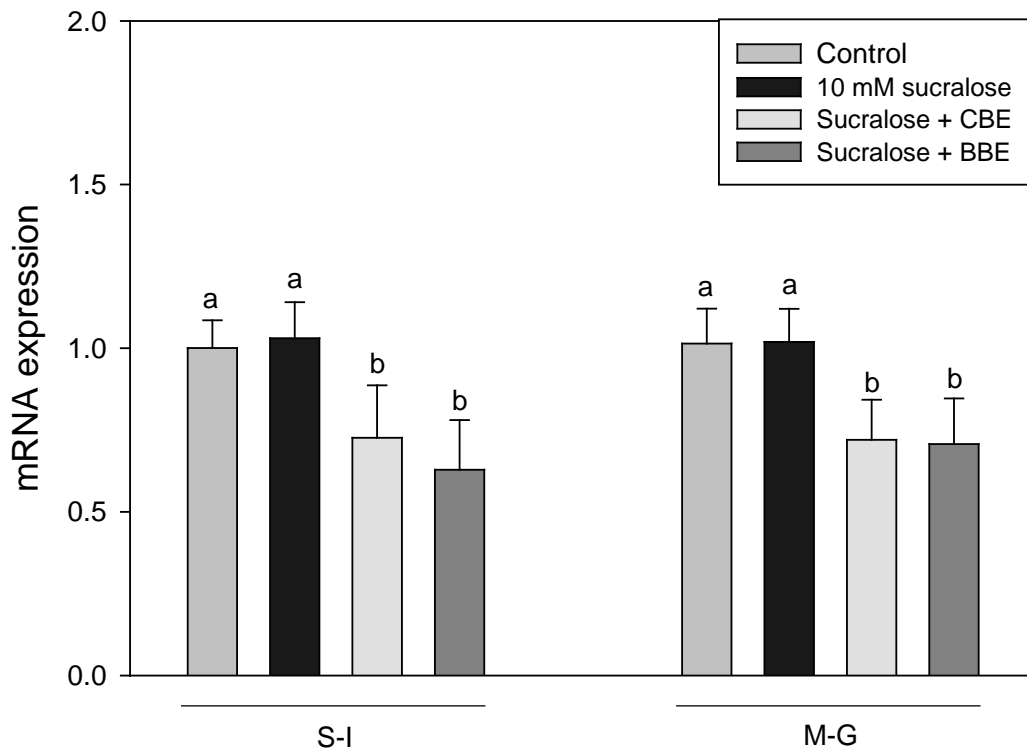


Figure 22. Effects of sucrose on carbohydrate digesting enzyme expression in the presence of sucralose. Human intestinal Caco-2 were treated with 200 $\mu\text{g/mL}$ of CBE or BBE in presence of sucralose (10 mM) for 1hr. S-I and M-G mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts. CBE: Cranberry extracts, BBE: Blackberry extracts.

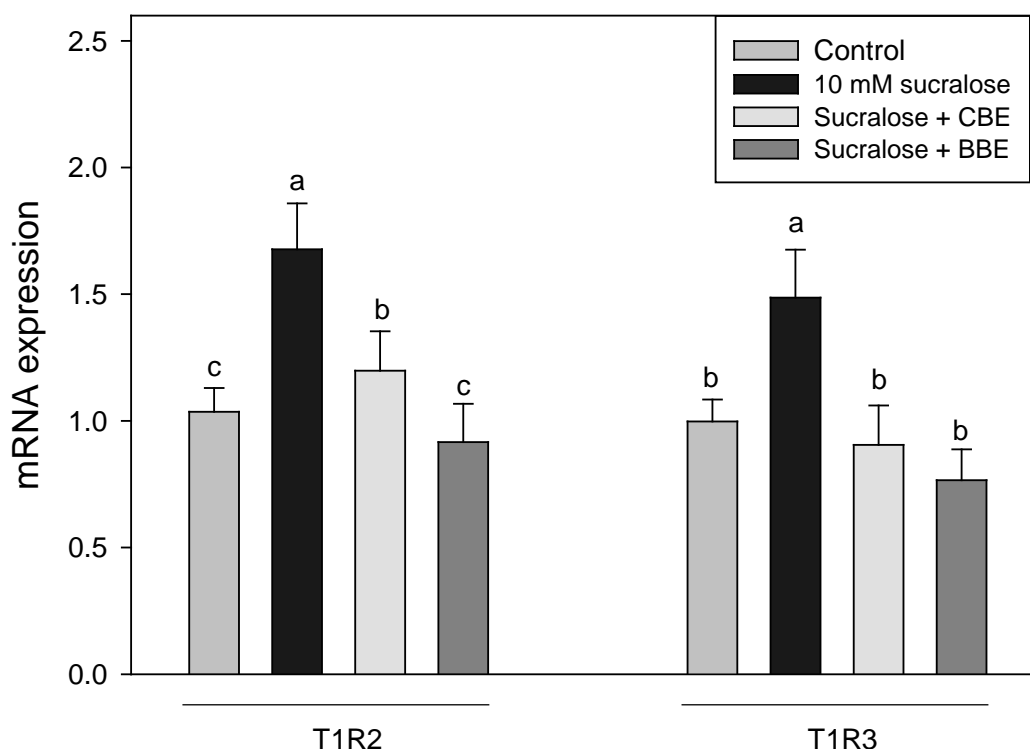


Figure 23. Effects of sucrose on sweet-sensing receptors expression in the presence of sucralose. Human intestinal Caco-2 were treated with 200 $\mu\text{g/mL}$ of CBE or BBE in presence of sucralose (10 mM) for 1hr. T1R2 and T1R3 mRNA levels were normalized to GAPDH as housekeeping genes. Results are presented relative value compare to the control groups as mean \pm SD of three independent experiments (n=3). Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's test. CBE: Cranberry extracts, BBE: Blackberry extracts.

3.4.9 Conclusion

In the current study, cranberry and blackberry extracts were evaluated for potential benefits against postprandial hyperglycemia, a key metabolic feature of T2DM and its complication. Our finding showed significant evidence of CBE and BBE in lowering plasma levels after a meal. In particular, we found that both CBE and BBE reduced glucose uptake in the presence of sucrose and sucralose in human intestinal Caco-2 cell line. The ability of CBE and BBE to prevent sucrose and sucralose-induced intestinal glucose uptake is associated with inhibition of α -glucosidase activity as well as gene expression. Notably, BBE with higher total phenolic contents than CBE showed α -glucosidase inhibitory activity with no effects on glucose transporters, while CBE showed inhibitory actions on both. These results show that certain berries exhibit more potential for the prevention of postprandial hyperglycemia than others, indicating that specific profile of nutrients and/or phenolic compounds present in different berries is of importance for the observed cellular effects.

Current findings also suggest the involvement of sweet-sensing receptors as potential upstream targets that regulate glucose transport and uptake. Taken together, our results suggest that increased intake of berries may be a promising strategy to delay postprandial glucose flux into the system and has important implications in the prevention of T2DM & its complications. However, the potential benefits of berry extracts need to be verified in *in vivo* and *clinical* studies in order to confirm the physiological relevance of current findings.

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